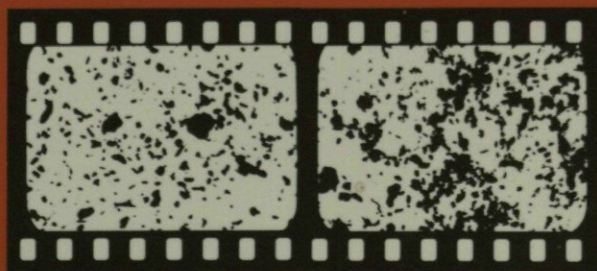# STRUCTURE AND STABILITY OF
METHANOGENIC GRANULAR SLUDGE

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Structure and stability of methanogenic granular sludge

Proefschrift

ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
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CONTENTS

Chapter 1

INTRODUCTION

BIOMASS RETENTION IN ANAEROBIC WASTE WATER TREATMENT

Interest in anaerobic wastewater treatment was greatly enhanced when energy prices increased and more stringent legislation for industrial waste waters was introduced in the early seventies. Additionally, research in anaerobic wastewater treatment was stimulated because oxygen transfer in aerobic systems demanded high energy inputs and large amounts of surplus sludge were produced in aerobic purification processes. A main drawback of anaerobic treatment at that time was the long wastewater retention time which had to be applied in conventional anaerobic systems to achieve an acceptable waste removal efficiency. This problem was overcome by the development of new reactor configurations in which the anaerobic biomass was retained. Several systems were developed like the Anaerobic Filter (Young and McCarty, 1969), Upflow Sludge Blanket (USB) reactor (Lettinga et al., 1980), Fluidized Bed Reactor (Heijnen, 1984), baffled reactor (Bachmann et al., 1985), and Anaerobic Gas Lift Reactor (Beeftink and van den Heuvel, 1987a). Extensive reviews on anaerobic systems based on biomass retention were presented by Henze and Harremoes (1983), van den Berg (1983), Speece (1983), Switzenbaum (1983, 1988), and Heijnen et al., (1986).

The most widely applied anaerobic reactor type in which solids retention time is uncoupled from hydraulic retention time is the Upflow Anaerobic Sludge Blanket (UASB) reactor. In this reactor biomass is retained by self immobilization of anaerobic bacteria into granular sludge. At present more than 80 full scale UASB reactors are in operation (de Zeeuw, 1988), and a variety of waste waters are treated satisfactorily in UASB reactors (Hulshoff Pol, 1989).

Below a short overview of the anaerobic processes occurring in anaerobic methanogenic environments is given. Thereafter, some mechanisms which are of importance in the immobilization of methanogenic biomass are discussed. Finally the outline of this thesis is presented.

ANAEROBIC MICROBIAL DEGRADATION PROCESSES

The complete anaerobic degradation is characterized by distinct processes in which several physiological types of bacteria are involved, (Gujer and Zehnder, 1983, Stronach et al., 1986, Pavlostathis and Giraldo-Gomez, 1991). Particulate organic material is hydrolyzed to organic monomers like sugars, amino acids and long chain fatty acids. Subsequently, monomers are fermented to acetate, carbon dioxide, hydrogen and reduced products such as alcohols, lactate and fatty acids (e.g. propionate, butyrate). Reduced organic products are converted to the methanogenic substrates acetate and hydrogen (Fig. 1). All these conversions are carried out by specialized bacteria, and therefore a complex microbial population is present in granular sludge (Fig. 2). Dolfing (1987) showed that granular sludge can be obtained after cultivation in laboratory UASB reactors on ethanol and propionate as sole carbon and energy source. With these substrates a reduced complexity of the microbial population is expected. Work presented in this thesis mainly deals with granules grown under defined conditions with ethanol and propionate as substrates.

CONVERSION OF ETHANOL AND PROPIONATE

Ethanol is a versatile substrate as complete conversion of ethanol to methane and carbon dioxide can proceed via several reaction sequences. Possible ways of degrading ethanol are presented in Table 1. Direct conversion to methane and acetate (equation 1) is carried out by *Methanogenium organophilum* (Widdel et al., 1988). In anaerobic environments the conversion of ethanol to acetate and hydrogen may be the most important reaction (equation 2) (Dubourguier et al., 1986). This reaction was first recognized by Bryant et al. (1967) who isolated the ethanol oxidizing S-organism from a previously believed pure culture of *Methanobacillus ·omelianskii*. This reaction can also be carried out by *Pelobacter carbinolicus* (Schink, 1984), a bacterium which was found to be present in high numbers in anaerobic sludges from digesters treating easily fermentable carbohydrates (Dubourguier et al., 1986).

Ethanol is degraded to acetate as sole product by the homoacetogenic bacteria *Clostridium aceticum* (Braun et al., 1981) and *Clostridium formicoaceticum* (Andreesen et al., 1970). The homoacetogenic reaction of ethanol is independent of the hydrogen concentration (equation 3, Table 1). However, these homoacetogenic bacteria are also able to convert hydrogen and carbon dioxide to acetate at high hydrogen concentrations (equation 14, Table 1). In well operating reactor systems at low hydrogen partial pressures these bacteria will convert ethanol to acetate, whereas in disturbed systems at high hydrogen concentrations acetate will be formed from

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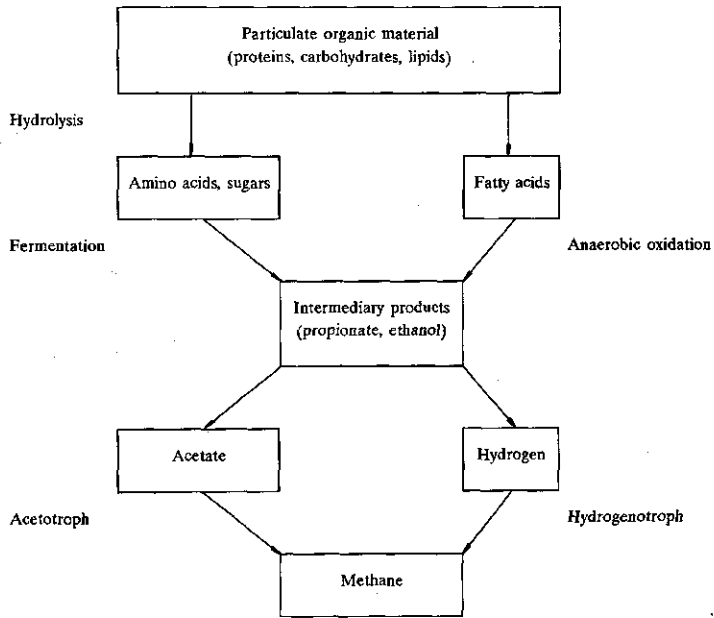
FIG. 1. Anaerobic digestion of particulate organic material to methane and carbon dioxide.
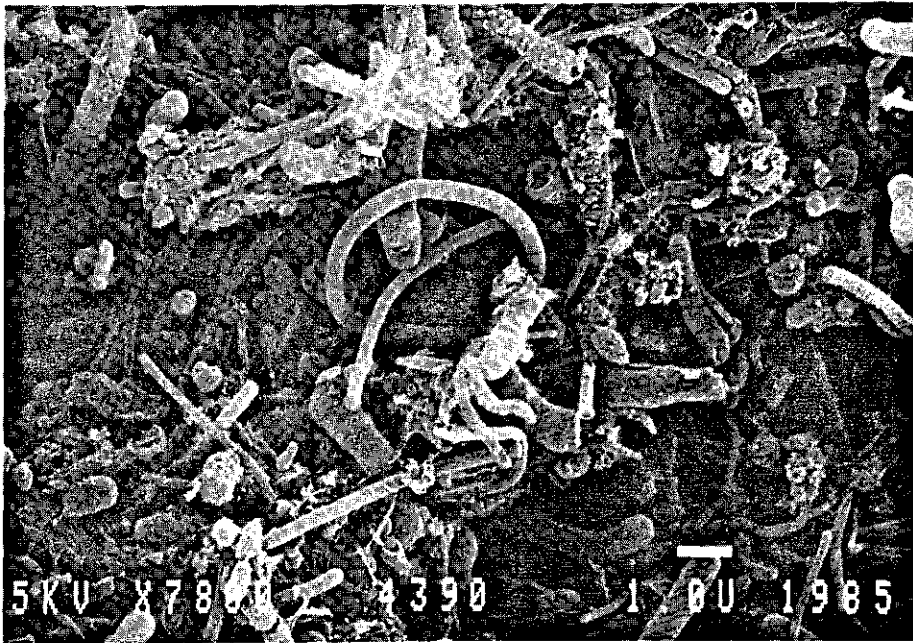
FIG. 2. Scanning electron photomicrograph of the complex microbial population in methanogenic granular sludge grown on waste water of a liquid sugar plant (CSM, Breda). Bar = 1 μm.

	Reactions	$\Delta G^{\circ\prime}$ kJ/reaction
1	$CH_3CH_2OH + 0.5HCO_3^- \longrightarrow CH_3COO^- + 0.5CH_4 + 0.5\,H_2O + 0.5H^+$	- 58.2
2	$CH_3CH_2OH + H_2O \longrightarrow 2H_2 + CH_3COO^- + H^+$	+ 9.6
3	$CH_3CH_2OH + HCO_3^- \longrightarrow 1.5CH_3COO^- + H_2O + 0.5H^+$	- 42.7
4	$CH_3CH_2OH + 2HCO_3^- \longrightarrow CH_3COO^- + 2CH_3CH_2COO^- + 3H_2O + H^+$	- 123.4
5	$CH_3CH_2OH + CH_3COO^- \longrightarrow CH_3CH_2CH_2COO^- + H_2O$	- 38.5
6	$CH_3CH_2COO^- + 3H_2O \longrightarrow CH_3COO^- + HCO_3^- + 3H_2 + H^+$	+ 76.1
7	$CH_3CH_2COO^- + HCO_3^- + H^+ + 3H_2 \longrightarrow CH_3CH_2CH_2COO^- + 3H_2O$	- 76.1
8	$CH_3CH_2COO^- \longrightarrow 0.5CH_3COO^- + 0.5CH_3CH_2CH_2COO^-$	0
9	$CH_3CH_2COO^- + 1.8H_2O \longrightarrow 0.8CH_3COO^- + 0.2CH_3CH_2CH_2COO^- + 0.6HCO_3^- + 1.8H_2 + 0.6H^+$	+ 45.8
10	$CH_3COO^- + H_2O \longrightarrow CH_4 + HCO_3^-$	- 31.0
11	$CH_3COO^- + 4H_2O \longrightarrow 2HCO_3^- + 4H_2 + H^+$	+ 104.6
12	$4H_2 + HCO_3^- + H^+ \longrightarrow CH_4 + 3H_2O$	- 135.6
13	$4CHOO^- + H_2O + H^+ \longrightarrow CH_4 + 3HCO_3^-$	- 130.4
14	$4H_2 + 2HCO_3^- + H^+ \longrightarrow CH_3COO^- + 4H_2O$	- 104.6

carbondioxide and hydrogen (Dolfing, 1988).

Conversion of ethanol to propionate was reported for *Desulfobulbus propionicus* (Laanbroek et al., 1982), *Pelobacter propionicus* (Schink, 1984) and a non-classified propionigenic strain X-4 (Samain et al., 1982) (equation 4, Table 1). The pathway of propionate formation from ethanol was elucidated for *Desulfobulbus propionicus* (Stams et al., 1984) and *Pelobacter propionicus* (Schink et al., 1987). Externally provided hydrogen had only a slight effect on the conversion of ethanol by *Pelobacter propionicus*, whereas hydrogen inhibited the conversion of ethanol to propionate of *Desulfobulbus propionicus*. The reason for the inhibitory effect of hydrogen on the ethanol conversion by *Desulfobulbus propionicus* is not known (Schink et al., 1987).

In comparing the pathways of anaerobic ethanol degradation Schink et al. (1985) observed that upto 30% of ethanol was degraded through propionate in Knaack Lake sediment and in anoxic sewage sludge. Addition of hydrogen to Knaack Lake sediment inhibited ethanol degradation drastically and led to a significant accumulation of butyrate (equation 5, Table 1), indicating a similar ethanol conversion as described for *Clostridium kluyveri* (Bornstein and Barker, 1948). However, butyrate did not accumulate under in situ conditions (Schink et al., 1985).

For propionate conversion to methane and carbon dioxide an initial reaction was described in which propionate is oxidized to acetate and hydrogen (equation 6, Table 1). Only a few obligate syntrophic acetogenic bacteria have been described which can carry out this reaction. At present *Syntrophobacter wolinii* in coculture with *Desulfovibrio* G11 is the only defined biculture able to oxidize propionate (Boone and Bryant, 1980). The obligate syntrophic organism *Syntrophobacter wolinii* only converts propionate to acetate, carbon dioxide and hydrogen (or formate) in the presence of a hydrogen consuming bacterium. Upto now it is not possible to grow this bacterium in pure culture, whereas other obligate proton reducing bacteria such as the butyrate oxidizing *Syntrophomonas wolfei*, was obtained in pure culture with crotonate (Beaty et al., 1987). In an enriched methanogenic propionate degrading coculture the most numerous propionate oxidizing bacterium was rod shaped and non-motile (Koch et al., 1983). This enrichment could be grown with sulfate reducing bacteria or methanogens (Houwen et al., 1990a). However, unlike *Syntrophobacter wolinii* which is Gram positive, the propionate oxidizing bacterium stained Gram-negative (Houwen et al., 1987, 1990a).

Recently Tholozan et al. (1988, 1990) observed a reductive carboxylation of propionate to butyrate in batch experiments with a methanogenic enrichment culture. Several reactions were proposed for this phenomenon (equation 7, 8, 9, Table 1). At present it is still unclear which role the reductive propionate conversion plays in natural ecosystems (Tholozan et al., 1988).

Except the conversions of ethanol and propionate described above, other

reactions may occur under non steady state conditions. In batch experiments with methanogenic granular sludge butyrate, iso-butyrate, butanol, propionate and propanol were formed from ethanol, and butyrate, valerate and iso-valerate were produced from propionate (Grotenhuis et al., 1986).

METHANOGENESIS

Methanogenesis from hydrogen, formate and acetate, which are the main products from ethanol and propionate conversion in well operating reactor systems, are discussed below.

CONVERSION OF HYDROGEN AND FORMATE

Methanogenesis from hydrogen (equation 12, Table 1) and formate (equation 12, Table 1) is highly favourable under standard conditions and can be carried out by a large variety of methanogenic bacteria. The bacteria which are able to convert hydrogen and/or formate to methane are classified in three orders of methanogens namely *Methanobacteriales*, *Methanococcales* and *Methanomicrobiales*. Interested readers should refer to several comprehensive reviews upon these classifications (Zeikus, 1977, Balch et al., 1979, Zehnder et al, 1981, Dubach and Bachofen, 1985, Jones et al. 1987, and Vogels et al. 1988).

The role of hydrogen as an essential intermediate in the conversion of ethanol to methane was first recognized by Bryant et al. (1967) who showed that the culture *Methanobacillus omelianskii* isolated by Barker in 1936 really consisted of an association of two different microorganisms. One organism was the non methanogenic "S" organism, which oxidized ethanol to acetate plus hydrogen (equation 2, Table 1), while the other bacterium *Methanobacterium bryantii* (formerly called *Methanobacterium* strain MoH), reduced the bicarbonate present in the medium with the hydrogen produced by the S organism (equation 12, Table 1). These findings as well as the demonstration of hydrogen as an intermediate in the rumen (Hungate, 1967) led to the concept of 'interspecies hydrogen transfer'(Iannotti et al., 1973). Within this concept a coupling occurs of a hydrogen producing reaction which becomes unfavourable at high hydrogen partial pressures (equation 2, 6, 9, 11, Table 1) and a hydrogen consuming reaction (equation 12, Table 1).

After the recognition by Bryant et al. (1967) of interspecies hydrogen transfer for ethanol conversion, similar processes were described for butyrate (McInerney et al. 1979, 1981, Dwyer et al., 1988, Ahring et al., 1987a, b, 1988), propionate (Boone and

Bryant, 1980), acetate (Lee et al., 1988a, b), benzoate (Mountfort et al., 1984, Dolfing and Tiedje, 1988), higher fatty acids (Stieb and Schink, 1985, Roy et al., 1986), phenol (Knoll and Winter, 1989) and chlorinated benzoates (Dolfing and Tiedje, 1986, Krumme and Boyd, 1988). Proton reducers depend on the presence of methanogens. However, some could also be obtained in pure culture. The ethanol degrader *Pelobacter carbinolicus* grows well in pure culture with acetoin (Schink et al., 1984, Dubourguier et al., 1986). Butyrate-degraders can grow in pure culture on crotonate (Beaty et al., 1987, Lorowitz et al., 1989) and unsaturated short chain fatty acids (Amos and McInerney, 1990). Attempts to grow propionate oxidizers in pure culture failed up to now (Houwen, 1991). Pure cultures are advantageous for both physiological and ecological studies (Beaty et al., 1989, Zhao et al., 1990).

Interspecies transfer of molecular hydrogen from obligately proton-reducing or facultative fermenting bacteria to chemolithotrophic methanogens is thought to be most important in methanogenic systems with low concentrations of intermediates. As ethanol and propionate conversion are of importance in the study of this thesis some more details are given upon the effect of interspecies hydrogen transfer on the microbial conversion of these substrates.

For the acetogenic ethanol oxidation coupled to proton reduction (equation 2, 12, Table 1) a hydrogen partial pressure below 10^{-1} atm is required. Degradation of propionate to acetate and hydrogen is only possible below 10^{-4} atm (Fig 3., Gujer and Zehnder, 1983, McCarty and Smith, 1986). These low hydrogen partial pressures in methanogenic systems are created by the chemolithotrophic methanogens.

The effect of interspecies hydrogen transfer on ethanol and propionate conversion was studied by Smith and McCarty (1989) in perturbed continuously stirred tank reactors. Both substrates can be converted only at negative ΔG values which is the case for propionate at hydrogen partial pressures below 10^{-4} atm whereas for ethanol conversion a much higher hydrogen pressure upto 10^{-1} atm is allowed (Fig. 3.). Smith and McCarty (1989) studied the kinetic behaviour of an ethanol and propionate fed reactor in steady state by a sudden addition of a high dose of ethanol to the reactor followed by measuring the hydrogen partial pressure and the conversion of ethanol and propionate. After the ethanol dose they observed an accumulation of propionate, whereas the ethanol degradation continued after the extra ethanol gift. These findings correspond well with thermodynamic considerations in which the propionate conversion is only possible at much lower hydrogen partial pressure than the conversion of ethanol (equation 6, 2, Table 1).

From the combination of the thermodynamics and kinetics of the ethanol and propionate conversion the maximal distance between the hydogen producing and hydrogen consuming bacteria can be calculated (Gujer and Zehnder, 1983, McCarty and Smith, 1986). This maximal distance was calculated to be 63 μm for ethanol conversion at a mean H_2 residence time of $3.4 * 10^{-1}$ s and 2.6 μm for propionate
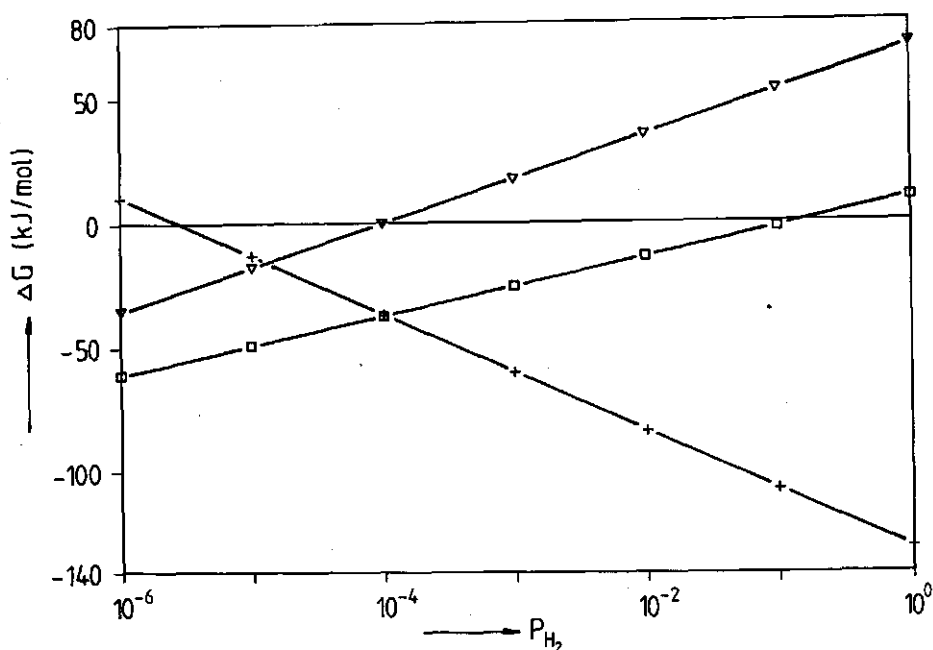
FIG. 3. Amount of free energy as function of the hydrogen partial pressure for the conversion of propionate, ethanol and hydrogen as given in table 1 for reactions 6 (∇), 2 ($\square$) and 10 (+). Concentrations of ethanol, propionate and acetate are assumed to be 30 mM and HCO_3^- 100 mM.

conversion at a mean H_2 residence time of $5.6 * 10^{-4}$ s (Grotenhuis et al., 1990). The distance of only 2.6 μm for propionate degradation led to the hypothesis of the spatial orientation of bacteria in which propionate oxidizing bacteria and hydrogen consuming bacteria are close together. For ethanol oxidizing bacteria no such orientation is expected since the maximal distance of 63 μm suggests that short distances between the ethanol oxidizing bacteria and hydrogen consuming bacteria are not essential for ethanol conversion (Grotenhuis et al., 1990, Stams et al., 1989).

Recently discussions arose whether hydrogen or formate is the most important electron carrier in syntrophic degradations (Thiele and Zeikus, 1988a, b, c, Ozturk et al., 1988) (equation 13, Table 1). Hydrogen is poorly soluble in water. At 37°C a hydrogen partial pressure of 1 atm the dissolved concentration of hydrogen is about 0.6 mM (Boone et al., 1989). It can be calculated that at the same $\Delta G'$ value the hydrogen concentration in the liquid is 260 fold lower than the formate concentration. This results in a 98-fold faster mass transfer rate of formate (Boone et al., 1989), despite the higher diffusion coefficient of hydrogen of $4.5 * 10^{-5}$ cm^2/s versus $1.5 * 10^{-5}$ cm^2/s for formate (Cussler, 1985). The role of formate as intermediate is supported by the fact that the hydrogen oxidation rate of *M. formicicum*, the prevalent hydrogenotrophic methanogen in a digestor system, was

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too low to account for the observed rate of methanogenesis (Thiele et al., 1988c). Thiele and Zeikus (1988c) also reported the formation of ^{14}C-formate from $^{14}CO_2$ in a syntrophic ethanol-degrading coculture. Formate can be used by many hydrogenotrophic methanogens as a substrate (Zeikus, 1977, Zehnder et al, 1981, Jones et al. 1987, and Vogels et al. 1988). In addition, some proton-reducing bacteria are able to form formate (Thiele and Zeikus, 1988a, Boone et al., 1989). In suspended cultures formate transfer may prevail, since the distance between the syntrophic partners is too large and the diffusion coefficients of hydrogen are too low to explain measured methane production rates (Thiele and Zeikus, 1988a, Ozturk et al., 1989, Boone et al., 1989). However, in methanogenic granular sludge with a high biomass density, hydrogen may be the most important electron carrier, since the distances between the hydrogen producing and hydrogen consuming bacteria are small enough to explain measured degradation rates by transfer of hydrogen (Stams et al., 1989, Grotenhuis et al., 1990). In addition, in propionate grown granular sludge microcolonies were observed consisting of propionate oxidizing bacteria and methanogens immunologically related to *Methanobrevibacter arboriphilus* AZ (Grotenhuis et al., 1990). This methanogen is not able to grow on formate (Zehnder and Wuhrmann, 1977). Also in some syntrophic ethanol and butyrate-degrading cultures formate transfer can be excluded, because the methanogens present in these cultures are unable to use formate (Bryant et al., 1967, McInerney et al., 1981, Ahring and Westermann, 1987a, b).

The interconversion of formate to hydrogen and carbon dioxide, and vice versa observed by Wu et al. (1991) suggests that both formate and hydrogen function as electron carrier in syntrophic degradations.

CONVERSION OF ACETATE

Acetate accounts for about 70% of the methane formed from organic matter, whereas the other 30% is formed from H_2/CO_2 (Gujer and Zehnder, 1983, Mah et al., 1978). Acetate is a growth substrate of mesophilic and thermophilic methane bacteria (equation 10, Table 1). Under thermophilic conditions acetate can also be degraded by syntrophic consortia (equation 11, 12, Table 1) (Zinder and Koch, 1984, Lee and Zinder, 1988a, Zinder, 1988).

The first acetate degrading bacterium which was obtained in pure culture was *Methanosarcina barkeri* (Schnellen, 1947). Thereafter, other *Methanosarcina* species were isolated including some thermophilic strains (Vogels et al., 1988, Ollivier et al., 1984, Zinder et al., 1985, Zinder and Mah, 1984, Touzel et al., 1985, Clarens and Moletta, 1990).

Isolation of filamentous aceticlastic methanogens was not reported until 1980

(Zehnder et al., 1980). One of the reasons for the late isolation of *Methanothrix* *soehngenii*, was its low growth rate of 0.0032 h^{-1} (Zehnder et al., 1980, Huser et al., 1982). Isolation was also difficult because *Methanothrix soehngenii* did not grow well in suspension and not at all on solid media (Zehnder et al., 1980). Later, several other strains of *Methanothrix* were described including some thermophilic strains. The thermophilic strains do not form long filamentous rods (Nozhevnikova and Chudina, 1985, Zinder et al., 1987). Although the physiological and morphological properties of the mesophilic *Methanothrix* strains are comparable (Touzel et al., 1988), *Methanothrix concilii* was renamed *Methanosaeta concilii* (Patel and Sprott, 1990). An unnamed rod-shaped acetoclastic thermophilic methanogen was isolated by Ahring and Westermann (1984, 1985). Besides acetate this thermophile uses also hydrogen and formate for growth.

Besides the different shape and substrate spectrum of *Methanosarcina* and *Methanothrix* the affinity of these strains for acetate differs remarkably. *Methanosarcina* cannot convert acetate below a threshold concentration of 0.2 mM, whereas the threshold for *Methanothrix* is 0.01 mM acetate. These differences in thresholds are due to the different enzyme systems for the activation of acetate (Jetten et al., 1990).

An obligate syntrophic acetate conversion was described for a thermophilic co-culture of *Methanobacterium* sp. strain THF and an acetate-oxidizing, rod-shaped bacterium (Zinder and Koch, 1984). Isolation of the acetate oxidizing bacterium from this co-culture was possible, because the bacterium appeared to be a homoacetogen which can grow on hydrogen and carbon dioxide (equation 14, Table 1) (Lee et al., 1988). The quantitative importance of syntrophic oxidation of acetate in anaerobic digestors is as yet unclear (Zinder, 1988).

IMMOBILIZATION OF BACTERIA

The immobilization of anaerobic bacteria into granular sludge is essential for the optimal performance of UASB reactors, in which the biomass retention time is highly exceeding the retention time of the waste water. Hulshoff Pol (1989) showed that environmental conditions like temperature, pH, type of waste water, and availability of nutrients had strong effects on the granulation process. The type of seed sludge and the process conditions applied during start-up were further of importance for immobilization of anaerobic bacteria. The chemical and bacteriological composition of methanogenic granular sludge was studied for granules grown on a waste stream of a liquid sugar plant (Dolfing, 1987). However, no information was available on the relative importance of the presence of extracellular polymers and inorganic precipitates, or of specific bacteria in granules. This lack of insight made a proper

immobilization of biomass with several waste waters still troublesome (Sayed et al., 1987).

Except in methanogenic granular sludge, microbial immobilization occurs in a large variety of microbiological habitats like biofouling on ships and in pipelines, formation of dental plaque, formation of activated sludge flocs, flocculation of brewing yeast, aggregation in cellular slime molds, and aggregation in myxobacteria. Several definitions dealing with microbial immobilization were agreed upon in the report of the Dahlem Konferenz on Microbial Adhesion and Aggregation (K.C. Marshall (ed), 1984). At this conference a microbial aggregate was defined as a collection of microbial cells in intimate contact (Calleja et al., 1984). Flocs, biofilms and granular sludge are all included in this definition although, a biofilm needs a substratum to which cells attach, whereas flocs and granules regularly do not.

The mechanism of adhesion of bacteria is thought to be the result of a sequence of events (Stronach et al., 1986, van Loosdrecht, 1988). First transport of cells to a surface or to each other can be realized by diffusive transport, convective transport (Characklis, 1981), or active transport. Then, the initial adhesion of bacteria occurs followed by firm attachment. Firm attachment may be realized by the formation of special cell surface structures in which extracellular polymers have shown to be essential for the development of surface films, but not for the initial adhesion of bacteria (Allison and Sutherland, 1987). As the firm attachment is realized, cell growth may occur and newly formed cells remain attached to each other leading to the formation of microcolonies or biofilms.

For aggregate formation, initial adhesion or adsorption of cells seems to be most critical (Characklis, 1989). Adhesion is defined in terms of energy involved in the formation of an adhesive junction. Two surfaces have adhered when energy is required to separate them (Rutter et al., 1984). The adhesion of bacteria to solid surfaces could be predicted by the height of charge and hydrophobicity of both surfaces (Loosdrecht et al., 1987a, b). During initial adhesion of bacteria to a substratum a thin adhesive film may be formed, leading to a more firm attachment. In case of bacterial adhesion to teeth this layer of salivary origin consisting of proteins is called an acquired pellicle (de Jong et al. 1984). Also other extracellular polymers are frequently observed in a wide variety of biofilms (Bryers 1987, Newman 1980). These polymers are thought to play an important role in the initial adhesion of bacteria to surfaces (Costerton et al. 1981, 1985, 1987, Characklis 1989) and in the stabilization of biofilms (Christensen and Characklis 1989). In the formation of microbial aggregates adhesion to inorganic precipitates may also play a role, because in experiments with an added amount of inorganic precipitates a more rapid start-up of UASB-reactors was found compared to experiments without such addition (Hulshoff Pol et al. 1983, Alibhai and Forster 1986, Mahoney et al. 1987).

OUTLINE OF THIS THESIS

Aspects concerning the structure and stability of methanogenic granular sludge are presented in this thesis. First an attempt was made for a chemical and bacteriological characterization of methanogenic granular sludge which was grown under defined conditions with simple substrates (Chapter 2, 3, 4, 5).

Chapter 2 describes the cultivation of methanogenic granular sludge with simple substrates in laboratory UASB-reactors under defined conditions. Chapter 3 deals with the localization and quantification of extracellular polymers in methanogenic granules. The role of calcium precipitates on size and strength of granular sludge is presented in Chapter 4.

The bacteriological structure and composition of granular sludge adapted to ethanol or propionate are discussed in Chapter 5. The presence of some bacteria in methanogenic granular sludge is discussed with respect to the conversion processes they carry out during anaerobic degradation.

Results on the surface properties of some isolates from granular sludge and some culture collection strains are presented in Chapter 6. One bacterium isolated from ethanol-grown sludge (*Clostridium granularum*) was characterized in more detail and was used for initial immobilization studies (Chapter 7).

Finally, the effect of substrate concentration on granule size of propionate grown granular sludge is presented in Chapter 8.

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Chapter 2

CULTIVATION OF METHANOGENIC GRANULAR SLUDGE UNDER DEFINED CONDITIONS

ABSTRACT

To study the structure and stability of granular sludge it is desirable to obtain methanogenic sludge which has been grown under well defined conditions. In research described in the following chapters granules were used which were obtained from two laboratory UASB reactors operated with either propionate and ethanol as substrates. The reactor configuration, inoculum and experimental procedure are described here.

INTRODUCTION

Formation of granular methanogenic sludge, consisting of densely packed anaerobic bacteria, was first observed in a 6 m^3 Upflow Anaerobic Sludge Blanket (UASB) reactor in 1974-1976 (de Zeeuw, 1988). Methanogenic granules developed well on waste water of a sugar factory and some other waste waters (Lettinga et al., 1980, Hulshoff Pol, 1989). The microbial structure of granular sludge is rather complex, as methanogenic degradation of organic matter requires consortia of specialized bacteria. Hulshoff Pol et al. (1983) and Dolfing (1987) showed that granule formation can be achieved with ethanol and mixtures of volatile fatty acids in lab scale UASB reactors. By using relative simple substrates a reduction in the complexity of the microbial population is to be expected. In addition, the use of simple substrates offers the possibility to study more fundamental aspects concerning the immobilization of anaerobic bacteria into methanogenic aggregates. The major part of the work described in this thesis deals with granular biomass grown with ethanol or propionate in two 5 l UASB reactors. The reactor configuration, inoculum and experimental procedure used to obtain defined granular sludge is described here.

MATERIAL AND METHODS

UASB reactor. Granulation was studied by Hulshoff Pol (1989) and Dolfing (1987) in perspex bench scale UASB reactors of 10 and 1 l respectively. Two identical glass UASB reactors (h = 70 cm, ø = 9.5 cm, V = 5 l) were built according to the design of Hulshoff Pol et al. (1983) and Dolfing (1987) (Fig. 1). To avoid a dead volume at the bottom of the reactor, a glass tube (ø = 1.2 cm) with two side openings (ø = 0.2 cm) at a height of 2 cm from the bottom was used as the influent point (1). Just below the flange (2) between the water jacket (3) and the top of the reactor (4) the reactor was narrowed to force the produced biogas into the gas/liquid/solid separator (5). The gas/liquid/solid separator (h = 24 cm, ø = 12 cm) was directly connected to the reactor. An inverted funnel (h = 20 cm, ø = 11 cm) in the separator collected the gas and kept the floating biomass in the reactor. Produced biogas left the reactor at the top via the inverted funnel (6). The liquid effluent left the reactor by overflow of the gas/liquid/solid separator via an effluent tube (ø = 2 cm) (7). The liquid effluent was sampled via a sample port at the top of the reactor (8).
If clogging at the reactor influent occurred, the influent tube was replaced via the crane (9). This crane avoided complete dismantling of the reactor in case of clogging. The water jacket (c) (h = 74 cm, ø = 13 cm) around the reactor was connected to a water bath and kept the reactor temperature at 35°C.
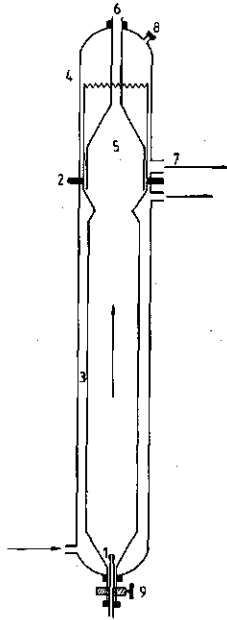
FIG. 1. Design of glass 5l UASB reactor. 1. influent tube, 2. flange, 3. water jacket, 4. reactor top, 5. gas/liquid/solid separator, 6. gas effluent, 7. effluent tube, 8. effluent sample port, 9. crane. Details of operation are given in the text.

Experimental procedure. The ethanol reactor was inoculated with approximately 100 ml granular sludge obtained from a 30 m^3 UASB reactor at a liquid sugar plant (CSM, Breda, The Netherlands) (Pette and Versprille, 1978). The propionate reactor was inoculated with 100 ml granular sludge cultivated by Dolfing (1987) in a 1 l UASB on a mixture of propionate and acetate for three to four months. This sludge came originally also from the same 30 m^3 reactor at the CSM.

The propionate reactor was operated at an influent concentration of 44.6 mM (5 gCOD/l) at a liquid retention time of 10 h, whereas the influent concentration of the ethanol reactor was maximal 26.0 mM (2.5 gCOD/l). At higher ethanol concentrations acidification of the medium occurred. The liquid upflow velocity in the 5 l reactors of 7,05 cm/h was between values reported by Hulshoff Pol (1983) and Dolfing (1987). Under these conditions both authors observed good granulation in their reactors.

The influent medium was prepared by mixing two concentrated solutions and anaerobic tap water in a thermostated (35°C) mixing vessel (V= 0.3 l) (Fig. 2.). In this manner the appropriate concentration of substrate was achieved. From the mixing vessel the medium was pumped into the UASB reactors at a rate of 500 ml/h. Gas production was measured by a wet gas meter (Schlumberger, Dordrecht, The

Netherlands) after removal of H_2S and CO_2 by leading the gas through concentrated solutions of ZnAc (2 N) and NaOH (5 N), respectively. The gas production was used as indicator for the functioning of the reactors. The substrate concentrations in the liquid effluent were used to determine the reactor efficiency. The effluent composition was measured daily by sampling at the sample port near the liquid overflow of the gas/liquid/solid separator. To avoid oxygen in the reactor the effluent left the reactor via an U-shaped tube. Sludge samples were taken by a sampling tube ($\emptyset = 1.2$ cm) made of glass, which was inserted in the reactor via the screwcap at the outlet of the inverted funnel at the top of the UASB.

Medium composition. Stock solution I contained (g/l): KH_2PO_4 0.76, $Na_2HPO_4.2H_2O$ 1.86, $NaHCO_3$ 4, $Na_2S.9H_2O$ 0.24, resazurin 0.001. Stock solution II contained (g/l): NH_4Cl 3, NaCl 3, $CaCl_2.2H_2O$ 2.1, $MgCl_2.6H_2O$ 1. The second stock solution also contained filter sterilized trace elements 1 ml/l and vitamins 1 ml/l. The trace elements solution contained (g/l): $FeCl_2.4H_2O$ 2, H_3BO_3 0.05, $ZnCl_2$ 0.05, $CuCl_2$ 0.03, $MnCl_2.4H_2O$ 0.5, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.05, $AlCl_3$ 0.05, $CoCl_2.6H_2O$ 0.05, $NiCl_2$ 0.05, ethylenediaminetetraacetate 0.5, and 1 ml concentrated HCl (Zehnder et al., 1980). The vitamin solution contained (mg/l): biotin 2, folic acid 2, pyridoxine hydrochloride 10, riboflavin 5, thiamine 5, nicotinic acid 5, pantothenic acid 5, vitamin B_{12} 0.1, p-aminobenzoic acid 5, thioctic acid 5 (Wolin et al., 1963). To stock solution I sodium propionate (43 g/l) or ethanol (12 g/l) were added as substrate. The concentrations in the final medium were diluted 10 fold with tap water. Concentrated media were heat sterilized in 10 l vessels; vitamins and trace elements were filter sterilized and added aseptically. All solutions were made anaerobic by flushing with N_2/CO_2 (4/1), and the atmosphere above the media was kept anaerobic using 10 l aluminium gas bags (Tesseraux Container GmbH, Dürstadt, Germany) filled with N_2/CO_2 (4/1). The pH of the influent was kept constant at pH 7.2 with 2N NaOH by a pH control unit (Radiometer, Zoetermeer, The Netherlands).

Activity measurements. Serum vials (V = 160 ml) were made anaerobic by flushing with N_2/CO_2 (4/1) and filled with 80 ml medium, previously deoxygenated by boiling and cooled to room temperature while gassing with O_2-free N_2/CO_2 (4/1). The medium contained (in g per l of demineralized water): KH_2PO_4 0.41, $Na_2HPO_4.2H_2O$ 0.53, NH_4Cl 0.3, NaCl 0.3, $CaCl_2.2H_2O$ 0.1, $MgCl_2.6H_2O$ 0.1, $NaHCO_3$ 4, resazurin 0.0005. Methanogenic granular sludge (200 - 700 mg VSS) was added to the vials. Subsequently the vials were sealed with butyl rubber stoppers and serum bottle caps. The vials were pressurized to 1.8 atmosphere with N_2/CO_2 (4/1). Under these conditions the pH of the medium was about 7.2. To remove traces of oxygen
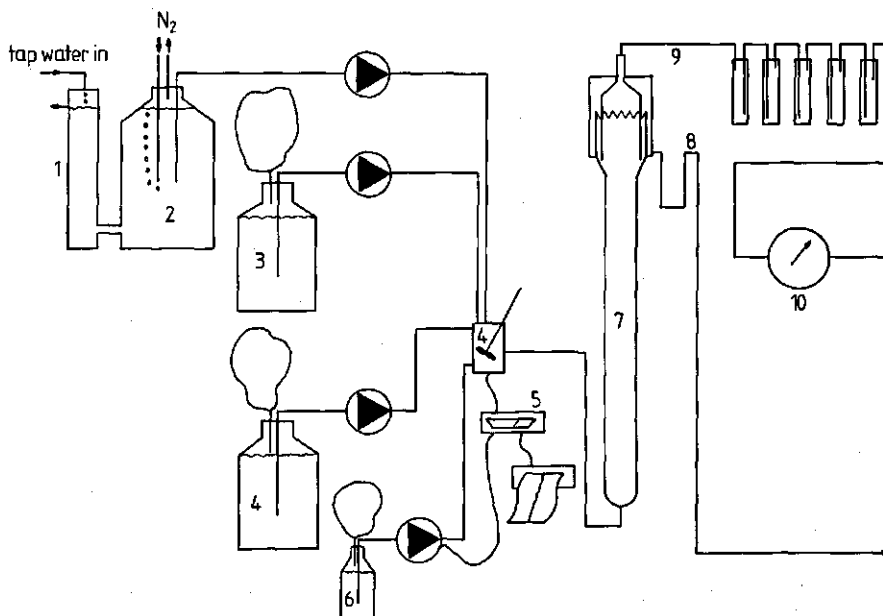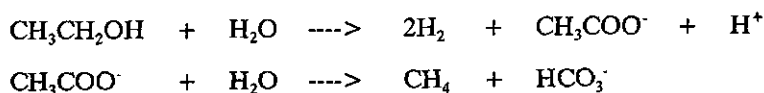
FIG. 2. Schematic diagram of the UASB reactor operation. 1. tap water overflow, 2. N_2 purge for anaerobic dilution water, 3. vessels with concentrated medium with gas bag to keep medium anaerobic, 4. mixing vessel, 5. pH control unit, 6. 2 N NaOH, 7. UASB reactor, 8. effluent, 9. gas wash bottles for CO_2 and H_2S removal (overflow bottle, water, NaOH (5 N), ZnAc (2 N) and liquid overflow 10. wet gas meter for CH_4 measurement.

$Na_2S.xH_2O$ (x = 7-9) was added by syringe to obtain a final concentration of 0.24 g/l. The granular sludge samples were preincubated overnight with 1 to 2 mM (0.1 to 0.2 g COD/l) propionate or ethanol, added from concentrated stock solutions. Substrate concentrations were increased to 20 mM (± 2 g COD/l) and the vials were shaken in a water bath at 35°C for 8 to 10 h. Liquid samples were taken with a syringe every 30 or 60 min, centrifuged and stored at -20°C for fatty acid analysis or analysed directly if ethanol was the substrate. At the end of the activity tests, the volume and dry weight of the granules were measured.

Analytical methods. Fatty acids and ethanol were determined by gas chromatography (Chrompack, Middelburg, The Netherlands) with a Chromosorb 101 column (Grotenhuis et al. 1991).

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TABLE 1. Conversion of ethanol to methane via acetate

$$CH_3CH_2OH \; + \; H_2O \; \text{---->} \; 2H_2 \; + \; CH_3COO^- \; + \; H^+$$

$$CH_3COO^- \; + \; H_2O \; \text{---->} \; CH_4 \; + \; HCO_3^-$$

RESULTS

Reactor performance. After inoculation both reactors were operated in batch mode with 20.0 mM (2.25 gCOD/l) propionate or 20.8 mM (2 gCOD/l) ethanol, respectively. Within one week gas production was observed in the reactors and operation at a liquid retention time of 10 h was started. The initial influent concentration was estimated from the maximum substrate conversion rate determined by actvity measurements with either propionate or ethanol. The initial influent concentration of the propionate reactor was 22.3 mM (2.5 gCOD/l), whereas the initial ethanol influent concentration was 1.3 mM (0.125 gCOD/l) leading to an organic loading rate of 53.6 mM propionate/$l_{reactor}$/d (6 gCOD/$l_{reactor}$/d) and 3.125 mM ethanol/$l_{reactor}$/d (0.3 gCOD/$l_{reactor}$/d), respectively (Fig. 3 and 4). Gas production is a sensitive indicator of the reactor operation, however fatty acid analysis proved to be more reliable to determine the COD removal in the reactors (Fig. 3 and 4).
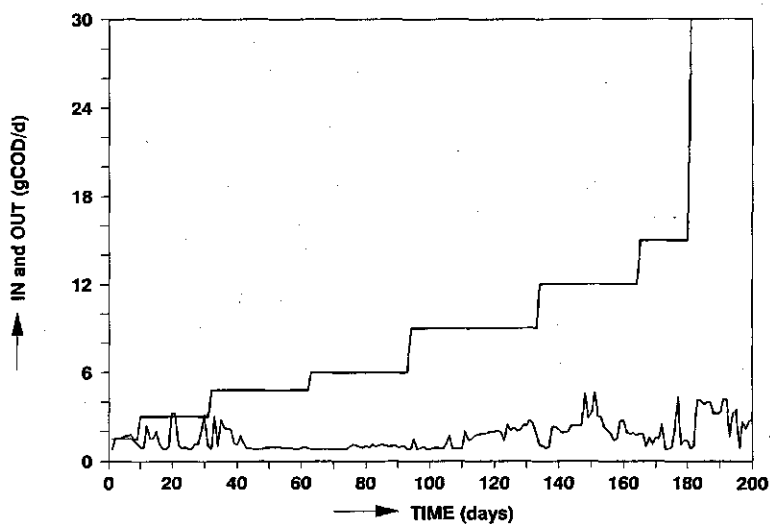
The influent concentration of the propionate reactor was lowered after 14 days as the substrate removal efficiency measured by fatty acid analysis in the effluent was below 80%. Thereafter, the influent concentration could be increased stepwise upto 89.3 mM (10 gCOD/l) with propionate removal efficiencies of more than 95% measured by fatty acid analysis of the effluent. Since UASB reactors are regularly operated for medium strength waste waters of about 44.6 mM (5 g COD/l) (de Zeeuw, 1984) the propionate concentration was lowered to this concentration for production of methanogenic granular sludge until the reactor was dismantled after three years of operation.

In the ethanol reactor an initial ethanol influent concentration of only 1.3 mM (0.125 gCOD/l) was applied because rapid conversion of ethanol to acetate led to an increase of the H^+ concentration leading to a drop in the pH (Table 1). Since the acetate decarboxylating methanogens in granular methanogenic sludge are sensitive to environmental changes a lowering of pH caused a decrease in acetate conversion (Gujer and Zehnder, 1983). The imbalance between ethanol oxidation and acetate conversion at high loading rates immediately led to a severe drop in pH. Severe drops of pH were often observed after switching the UASB reactor from batch mode operation to continuous operation as is shown in Fig. 5.

A maximum ethanol influent concentration of 26.0 mM (2.5 gCOD/l) could be maintained for maximal 6 months.
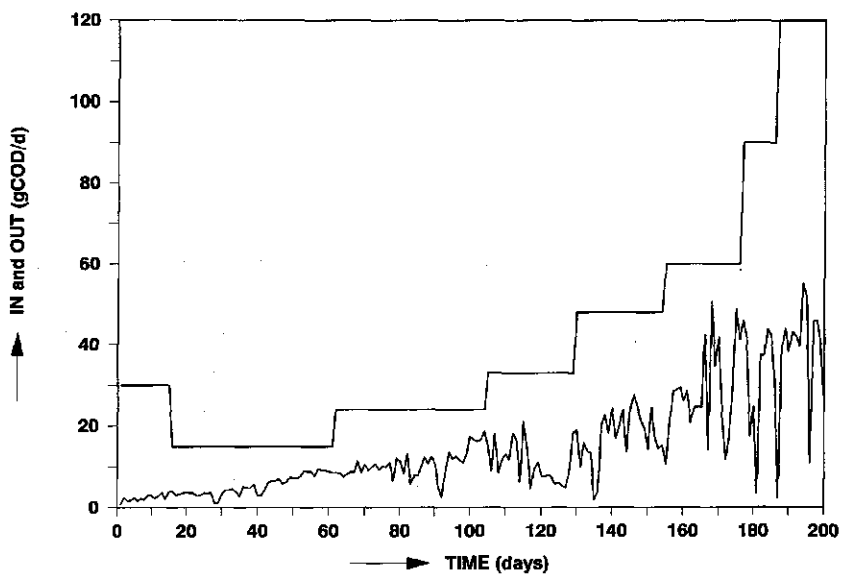
FIG. 3. Substrate influent concentration of laboratory UASB reactor fed propionate and gas production, used as indicator for well functioning of the reactor, as function of the operation time at a liquid retention time of 10 h. Maximum influent concentrations tested was 89.3 mM (10 gCOD/l) propionate.

FIG. 4. Substrate influent concentration of laboratory UASB reactor fed ethanol and gas production, used as indicator for well functioning of the reactor, as function of the operation time at a liquid retention time of 10 h. Maximum influent concentrations tested was 20 mM (2.5 gCOD/l) ethanol.
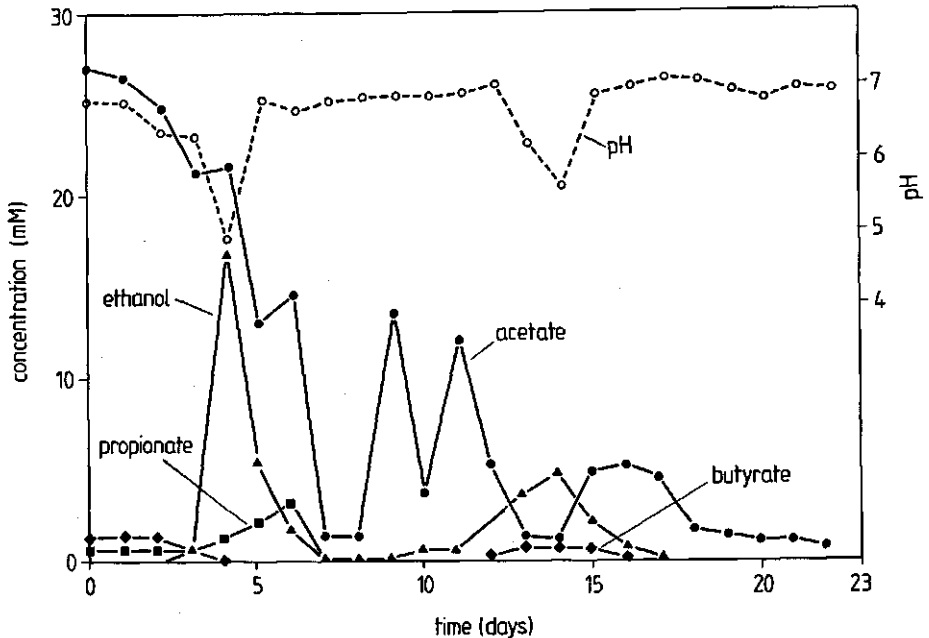
FIG. 5. Relative stability of an UASB reactor fed with ethanol. At time zero the reactor was changed from batch to flow through mode at 20 mM (2.5 gCOD/l) ethanol. This is the reason of the high initial acetate concentration. After each pH drop ethanol and acetate conversion was inhibited to a certain degree.

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Chapter 3

LOCALIZATION AND QUANTIFICATION OF EXTRACELLULAR POLYMERS IN METHANOGENIC GRANULAR SLUDGE

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SUMMARY

Extracellular polymers were localized and quantitatively analyzed in methanogenic granular sludge cultivated on either propionate or ethanol in laboratory Upflow Anaerobic Sludge Blanket (UASB) reactors. Electron microscopical analysis of ultrathin sections of the two sludge types stained with ruthenium red revealed the presence of extracellular polymers with different densities and structures. For quantification, granular sludge from a large scale UASB reactor at a liquid sugar plant, was also included in this study. A three step physical disintegration procedure was used to extract water soluble extracellular material from the granules. After each disintegration step the extracts were analyzed for polysaccharides and proteins. Cell damage and thus the contribution of intracellular proteins and polysaccharides was estimated simultaneously by the determination of free DNA and free ATP in the extracts. After two extraction steps up to 3.5 mg polysaccharides/g organic material and 5.5 mg protein/g organic material were extracted, whereas no significant increase of DNA was detected. The role of extracellular polymers in granular stability is discussed.

INTRODUCTION

Extracellular polymers are frequently observed in a wide variety of biofilms (Bryers 1987, Newman 1980). They are thought to play an important role in the initial adhesion of bacteria to surfaces (Costerton et al. 1987, Characklis 1989) and in the stabilization of biofilms (Christensen and Characklis 1989). The role of extracellular polymers (glycocalyx) in bacterial adhesion was extensively discussed by Costerton et al. (1985). Glycocalyx is defined as polysaccharide containing structures of bacterial origin lying outside the integral elements of the outer membrane of Gram-negative cells or the peptidoglycan of Gram-positive cells (Costerton et al., 1981). Extracellular acidic polysaccharides can be visualized by electron microscopy with ruthenium red staining (Luft 1971). Quantification of total extracellular polymeric adhesives is difficult to achieve, and therefore the importance of these polymers for the structure and stability of biofilms can only be estimated (White 1984).

Methanogenic granular sludge is a spherical biofilm. It consists of densely packed anaerobic microbial consortia. The granules can attain diameters of up to 5 mm (Lettinga et al. 1980). It is not clear whether extracellular polymers play a major role in the formation, structure and stability of these biofilms. Dolfing et al. (1985) compared several extraction methods for extracellular polymers from granular sludge and found 10 to 20 mg polysaccharide/g dry weight and upto 45 mg protein/g dry weight to be extracellular. However, as no experiments were included to correct for cell disrupture, intracellular polysaccharides could have been measured as well. In the experiments presented here cell disrupture was estimated by the determination of free ATP and free DNA.

To obtain more detailed information on the role of polymers in the structural features of granules, different types of granules have been investigated with electron microscopy and by chemical analysis. Results of these investigations are presented here.

MATERIALS AND METHODS

Granular sludge. Granular sludge was cultivated in 5 l UASB reactors with ethanol for 6 months or propionate for 30 months as substrates at a mean influent concentration of 26 or 44 mM, respectively. The liquid retention time was 10 h (Grotenhuis et al., 1991 a). The lab scale reactors were originally inoculated with granular sludge from a 30 m³ UASB reactor used at the Centrale Suiker Maatschappij (CSM) sugar refinery in Breda, The Netherlands (Pette and Versprille 1982). Sludge from the laboratory reactors was analyzed directly after sampling. For

comparison of the chemical composition, sludge from the 30 m³ reactor was also analyzed. The granules were collected and stored anaerobically at 4°C before analysis.

Electron microscopy. Granules were prefixed for 1 h at room temperature in 1.2% glutaraldehyde and 0.05% ruthenium red in 66 mM sodiumcacodylate buffer, pH 7.5 (Luft 1971). Free glutaraldehyde was removed by washing the granules for 10 min. in the same buffer with ruthenium red. Postfixation was done for 1 h in 0.33% OsO_4 and 0.033% ruthenium red in 66 mM cacodylate buffer (Luft 1971). After washing again in cacodylate buffer with ruthenium red, granules were dehydrated in graded ethanol series followed by transfer to propylene oxide and finally embedded in Epon resin. Ultrathin sections, prepared with an LKB (Bromma, Sweden) ultramicrotome using glass knives, were examined with a Philips EM 301 at 60 kV.

Preparation for quantitative analysis. To extract water soluble extracellular polysaccharides and proteins from the granular sludge, a 10 ml volume of granules was diluted with buffer solution to 40 ml. The buffer solution consisted of (g/l): Na_2HPO_4 0.7, $MgCl_2$ 0.1, and NaCl 2.5. Granules were gently homogenized with a 10 ml syringe (Becton Dickinson, Etten-Leur, The Netherlands) without a needle by continuously taking up and ejecting the suspension during 3 min. Part of this suspension (10 ml) was centrifuged (5 min, 13,000 x *g*) and the supernatant analyzed for extracellular compounds (fraction 1). The remaining 30 ml suspension was filled up to 40 ml with buffer. This suspension was homogenized further by passing it once along the plunger of a 15 ml Potter homogenizer (Tamson, Zoetermeer, The Netherlands). Subsequently, 10 ml of this suspension was centrifuged (5 min, 13,000 x *g*) and the supernatant analyzed (fraction 2). The remainder was homogenized another 9 times with the Potter homogenizer and 10 ml were centrifuged and the supernatant analyzed in the same way as fraction 2 (fraction 3). The remaining suspension (20 ml) was used to determine the total quantity of proteins, polysaccharides, ATP, DNA, dry weight and ash content (fraction 4).

Analytical methods. In all four fractions proteins, polysaccharides, ATP and DNA were quantified. Protein was measured according to Lowry et al. (1951) using bovin serum albumin as standard. For this determination, samples were first treated with 2 M HCl to remove sulfide and subsequently neutralized by an equal volume of 2 M NaOH.
The polysaccharide content was measured with the anthrone-sulfuric acid method (Dische 1962).
ATP was quantified with the luciferase assay according to Pradét (1967). Samples and ATP standards (0.5 ml) were boiled for 4 minutes in 5 ml of a solution

containing 50 mM Tris-HCl and 4 mM EDTA at pH 7.8. Then, 100 µl luciferine-luciferase reagent (ATP bioluminescence CLS, Boehringer, Mannheim, Germany) was added to 50 µl sample in polycarbonate cuvettes. The light emission was measured for 10 seconds in a photon counter (Biocounter M2010, Lumac/3M, Schaesberg, The Netherlands). A calibration curve treated in the same way as the standards, was made with ATP from 5 to 300 ng/ml.

For the analysis of total DNA in fraction 4, samples (10 ml) were passed through a French press (3 times, 50 bar). The extracts were incubated with 2% SDS and proteinase K (5 µl in 4 ml sample) for 20 min at room temperature. After centrifugation (5 min, 3,300 x g), DNA was precipitated by the addition of 0.4 ml of a 3 M sodium acetate solution and 14 ml 96% ethanol. This mixture was cooled to -70°C for 15 min. The DNA was purified further by centrifugation (15 min, 13,000 x g at 4°C), washing the pellet (70% ethanol), centrifugation and drying under vacuum. Total DNA was quantified after the formation of a specific and stable complex with 4',6-Diamidino-2-phenylindole·2HCl (DAPI) (Brunk et al. 1979). This complex can be quantified at 450 nm after excitation at 360 nm in a spectrophotofluorometer (Aminco-Bowman, American Instrument Company, Sliver Spring, Maryland 20910, U.S.A.).

The sludge volume was measured after settling of the granules in a metering glass. The volume between the granules was measured by the removal of the intergranular fluid by a syringe with a 0.1 mm diameter needle.

The approximate concentration of extracellular polymers in mg/ml extracellular space can be calculated by assuming that bacteria are identical spheres in a close-packed structure. The total volume occupied by n cells is $5.66r^3n$ (Durrant and Durrant 1970), leaving an extracellular space of 26% within the granules.

Dry weight and ash content of the granule suspension were determined after heating overnight at 105°C and reheating for 2 h at 600°C, respectively. The organic material was calculated by subtraction of the ash weight from the dry weight.

RESULTS

Electron microscopy

Different types of glycocalyx were found in ethanol and propionate-grown granular sludge after staining with ruthenium red and electron microscopical observation. In ethanol-grown granular sludge a variety of morphological types of bacteria was observed. At high magnification fine structured as well as roughly structured intercellular materials were distinguished by ruthenium red staining. Between colonies, a glycocalyx was found encapsulating cells, morphologically resembling *Methanosarcina* sp. (Fig. 1a). In the same sludge type roughly structured

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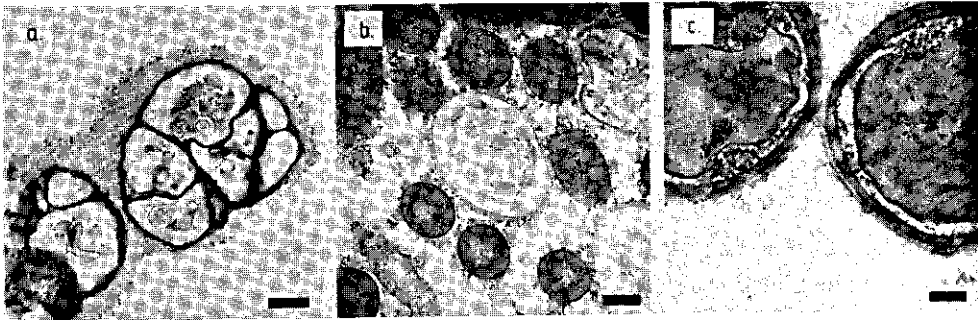
FIG. 1. Transmission electron micrographs of ruthenium red stained ultrathin sections of ethanol-grown granular sludge with several types of bacteria. a *Methanosarcina* sp. cells with intensely stained cell walls surrounded by electron translucent glycocalyx; bar = 0.5 μm. b Various unidentified bacteria connected by a network of roughly structured polysaccharides protruding from the cell wall; bar = 0.2 μm. c Bacteria attached to each other by fine structured polysaccharides covering a more electron dense cell wall layer. Note the fine fibrilar structures radiating from the cell wall surface; bar = 0.2 μm
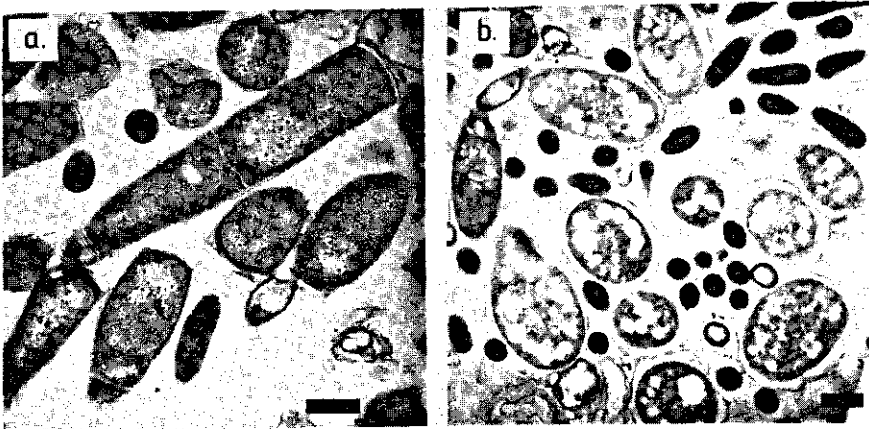
FIG. 2. Transmission electron micrographs of ruthenium-red stained ultrathin sections of propionate-grown granular sludge with micro-colonies of *Methanothrix* sp. showing a weak reaction of ruthenium red between the cells (a; bar = 0.5 μm) and a syntrophic culture of *Methanobrevibacter arboriphilus* AZ (electron dense) and a probably propionate oxidizing bacterium surrounded by ruthenium red stained zones (b; bar = 0.5 μm) embedded in Epon resin

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TABLE 1. Chemical composition of three types of granular sludge

Sludge	Polysaccharide	Protein	ATP	DNA	Ash
substrate	(% (wt) of organic material)				(% of dry weight)
CSM wastewater	12 ± 0.4	30.9 ± 1.6	0.006 ± 0.001	2.1 ± 0.19	10.0
ethanol	12.9 ± 0.3	35.7 ± 1.0	0.015 ± 0.005	1.3 ± 0.05	16.7
propionate	6.3 ± 0.5	32.4 ± 0.2	0.012 ± 0.001	3.1 ± 0.18	23.5

The values are given as percent of the total amount of organic material. CSM (Centrale Suiker Maatschappij, Breda, The Netherlands)

and ruthenium red stained extracellular polysaccharides protruded from the cell walls of two unidentified cell types which entangled each other (Fig. 1b). Direct attachment of cells was also observed (Fig 1c.).

Propionate-grown granular sludge predominantly consisted of two types of microcolonies, one consisting of the rod shaped bacteria which reacted with antisera raised against *Methanothrix soehngenii* (Grotenhuis et al., 1991 c). Within *Methanothrix* sp. colonies, material was present which only weakly stained with ruthenium red (Fig. 2a). The second type of microcolonies (Fig. 2b), is presumably composed of propionate oxidizing bacteria which are large and translucent surrounded by dark stained methanogens, immunologically related to *Methanobrevibacter arboriphilus* (DSM 744) (Grotenhuis et al., 1991 c). Ruthenium red stained zones were clearly observed around the large electron translucent bacteria.

Quantitative analysis of total polymers

   Results of the quantitative analysis of fraction 4 from CSM granular sludge, ethanol- and propionate-grown sludge are given in Table 1. The polysaccharide content of the methanogenic granular sludge grown on ethanol and of the CSM reactor was somewhat lower than the polysaccharide content of the average microbial cells (16.6%, Stouthamer, 1973). In propionate-grown granular sludge the amount of polysaccharides was even lower. The protein content in the granules was between 30 and 36%, which is also low compared to the 52.4% generally found in various bacteria (Stouthamer, 1973). The ATP levels in the granules were low as well, but the DNA content was as expected (3.2%, Stouthamer, 1973). The ash fractions were comparable to the findings of Dolfing et al. (1985) (10 to 20%) for CSM granular sludge.

Quantitative analysis of extracellular polymers

The amounts of extracted polysaccharides and proteins increased in the extracts with increasing disintegration forces (Table 2). Correction for cell disrupture was made by quantification of ATP and DNA in the extracts. In intact cells these components are only present intracellularly. The accuracy of the method used for ATP quantification was too low to measure the precise differences between the different steps of physical disintegration. Nevertheless, an increase in ATP after each disintegration step was found. Between fraction 1 and 2 the protein concentrations augmented significantly in all extracts and the polysaccharide concentrations increased for CSM and propionate grown granules. In fraction 2 only extracellular polymers were extracted. However, in fraction 3 some cells were probably damaged since DNA in the liquid increased two to threefold. In Table 3 the extracellular amounts of polysaccharides and proteins are given in four different ways for the three sludge types. For comparison data from Dolfing et al. (1985) are given, who used a more rigorous method for granule disintegration.

DISCUSSION

The presence of extracellular polysaccharides in methanogenic granular sludge was clearly demonstrated by the use of electron microscopy. Within the granules a large variety of ruthenium red stained polymer structures was observed. The concentrations of extracellular polymers in the extracellular space were 6 to 15 times lower than reported before (Dolfing et al., 1985). The summarized values of extracellular polysaccharides and extracellular proteins were 1.0 to 2.6 mg/ml granule of extracellular polymers in the three sludge types (Table 3). Stable gels of capsular polysaccharides were formed by *Rhizobium trifolii* at concentrations of 1 to 4 mg/ml with gel strengths of upto 1 N/cm^2 (Zevenhuizen 1984). The extraction method used in our study may have led to some underestimation of extracellular polymers; since small amounts of lysed cells are regularly observed by electron microscopy. These lysed cells can contribute to the release of DNA and therefore taken as cells damaged by the extraction procedure. In addition, material that remains attached to the cell wall or which is insoluble in water is not measured with our method. In any case, the extracellular polymer concentration in the intermicrobial space is high enough to form gels of sufficient strength to stabilize the granules to a certain extend. In propionate-grown sludge predominantly two types of microcolonies were found. One type consisted mainly of *Methanothrix soehngenii*, an organism which converts acetate in methane and carbon dioxide (Huser et al., 1982). This bacterium is often present in methanogenic granular sludge. Since this bacterium reacted only slightly with ruthenium red, its shape may stabilize the granules (Wiegant, 1988).

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TABLE 2. Extraction of polysaccharide, protein, and DNA from granular sludge in three steps of physical disintegration

Substrate sludge	Fraction	Polysaccharide	Protein	DNA
		(% extracted from total component)		
CSM wastewater	1[a]	1.65 ± 0.22	1.00 ± 0.05	1.60 ± 0.12
		*[b]	*	
	2	2.20 ± 0.01	1.77 ± 0.02	1.75 ± 0.04
	3	2.97 ± 0.08	2.83 ± 0.02	3.04 ± 0.22
ethanol	1	2.35 ± 0.47	0.93 ± 0.14	1.35 ± 0.33
			*	
	2	2.75 ± 0.60	1.51 ± 0.02	1.11 ± 0.08
	3	3.59 ± 0.10	2.29 ± 0.08	3.45 ± 0.01
propionate	1	1.77 ± 0.05	0.65 ± 0.01	1.83 ± 0.09
		*	*	
	2	2.23 ± 0.05	1.38 ± 0.03	1.67 ± 0.15
	3	3.52 ± 0.12	2.67 ± 0.01	3.74 ± 0.19

The values are given as percent of the total amount of the respective components
[a] Fractions were treated by (1) syringe, (2) syringe and once passing the plunger of a Potter tube and (3) syringe and a tenfold passing of the plunger of a Potter tube
[b] * indicates significant difference, whereas DNA had not changed significantly

TABLE 3. Concentration of extracellular polysaccharide and protein in three granular sludge types after physical disintegration of fraction 2 (1 x Potter homogeniser)

Sludge substrate	Biomass concentration	Extracellular polysaccharide				Extracellular protein			
	(mg org. mat./ml granule)[a]	(mg/g org. mat.)	(mg/g dry weight)	(mg/ml granule)	(mg/ml extra-cellular space)[b]	(mg/g org. mat.)	(mg/g dry weight)	(mg/ml granule)	(mg/ml extra-cellular space)[b]
CSM wastewater	66.9	2.6	2.4	0.18	0.83	5.5	5.0	0.37	1.72
ethanol	23.2	3.5	3.0	0.08	0.39	5.4	4.6	0.13	0.59
propionate	53.2	1.4	1.1	0.08	0.35	4.5	3.6	0.24	1.12
CSM[c]	38.5-45	11.1-26	10-20	0.5-1	2.34-4.69	50-58.4	45	2.25	10.6

[a] org. mat. means organic material.
[b] calculation based on the presence of 18% free water measured in CSM granular sludge and for the extracellular space a close-packed structure of cells was assumed.
[c] From Dolfing et al. (1985); CSM sludge characteristics: 10-23% ash, 10-20 mg extracellular polysaccharides/g dry weight, 45 mg extracellular protein/g dry weight and 0.05 g dry weight/ml granule.

Except extracellular polymers, also other factors may be involved in the stabilization of methanogenic granular sludge. The removal of calcium precipitates from granular sludge by EGTA, for example, led to a weakening or even complete disintegration of the granules (Grotenhuis et al., 1988, Grotenhuis et al., 1991 b).

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Chapter 4

EFFECT OF ETHYLENE GLYCOL-BIS(β-AMINOETHYL ETHER)-N,N-TETRAACETIC ACID (EGTA) ON STABILITY AND ACTIVITY OF METHANOGENIC GRANULAR SLUDGE

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SUMMARY

The effect of the calcium specific chelant ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid (EGTA) on methanogenic granular sludge from a laboratory scale upflow anaerobic sludge-blanket (UASB) reactor fed propionate and from a full scale reactor treating paper-mill waste-water was studied. Upon treatment with EGTA both sludge types showed a decrease in the calcium and phosphorus content and a release of protein and polysaccharides, leading to a decrease in strength of paper-mill granular sludge and a disintegration of propionate-grown granules. After treatment of propionate-grown granular sludge with high EGTA concentrations the methanogenic activity with propionate and acetate as test substrates decreased by 88 and 33%, respectively. The marked reduction in the propionate oxidation activity may be caused by a disruption of the special juxtapositioning of bacteria in the granules.

INTRODUCTION

Calcium is an important element in the aggregation of microbial biomass. It may function as flocculant by decreasing the surface charge of negatively charged microbes (van Loosdrecht et al. 1987) or be an essential constituent of the intercellular polymeric matrix (Fletcher 1980, Turakhia et al. 1983). Furthermore, the gel strength of extracellular matrices consisting of polysaccharides often depends on the concentration of calcium ions (Kang et al. 1982). Calcium precipitates such as calcium carbonate or hydroxyapatite may also serve as support material for the adhesion of bacteria (Arvin and Kristensen 1983, Harvey et al. 1984).

Attachment of bacteria to hydroxyapatite has been intensively studied in dental research (Newman 1980, Gibbons and van Houte 1975). The initial adhesion to hydroxyapatite is often mediated by a thin film of salivary origin consisting of proteins called an acquired pellicle (de Jong et al. 1984). Adhesion of bacteria to the acquired pellicle is likely to be stimulated by hydrophobic bonding due to hydrophobic amino acids associated with the cell wall (Nesbitt et al. 1982). Adsorption of extracellular polysaccharides to the acquired pellicle is enhanced by calcium ions (Rölla 1976). The positive charge of these ions is thought to interact with the negative surface charges of the polysaccharides and the pellicle. Treatment of bacterial cells attached to hydroxyapatite with the complexing agent ethylenediaminetetraacetate (EDTA) led to a partial removal of the cells (Newman 1980).

Methanogenic granular sludge which is formed in an upflow anaerobic sludge blanket (UASB) reactor consists of spherical biofilms of dense methanogenic biomass formed by self-aggregation of mixed microbial populations (Lettinga et al. 1980). Several studies on methanogenic granules have shown that calcium stimulates granule formation during the start-up of UASB reactors (Hulshoff Pol et al. 1983, Alibhai and Forster 1986, Mahoney et al. 1987). The exact mode of this stimulation is not clear. Therefore, a study was set up to investigate the role of calcium in stabilizing these methanogenic aggregates. Two sludge types with a high calcium content were selected (Grotenhuis et al. 1988). The effect of calcium removal with the specific chelant ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid EGTA on granule diameter, granule strength and methanogenic activity of these sludge types was studied.

MATERIALS AND METHODS

Granular sludge. Granular sludge originated from a propionate fed laboratory UASB reactor (Grotenhuis et al. 1988) and from a full scale UASB reactor (750 m^3) used for the purification of paper-mill waste-water (Papierfabriek Roermond, The Netherlands).

Media. A mineral salt solution consisting of 6 mM NH_4Cl and 110 mM NaCl (pH = 7.3, adjusted with 1 M HCl or 1 M NaOH) was used for EGTA treatment and subsequent determination of granule size and stability. Activity measurements were done in serum vials (vol. 160 ml) which were made anaerobic by flushing with N_2/CO_2 (4/1) and filled with 80 ml buffer solution, previously deoxygenated by boiling, and then cooled to room temperature under continuous gassing with O_2-free N_2/CO_2 (4/1). The buffer solution contained (in grams per litre of demineralized water): KH_2PO_4, 0.41; $Na_2HPO_4.2H_2O$, 0.53; NH_4Cl, 0.3; NaCl, 0.3; $CaCl_2.2H_2O$, 0.1; $MgCl_2.6H_2O$, 0.1; $NaHCO_3$, 4; resazurin, 0.0005.

EGTA treatment. Serum bottles of 1 l were filled with 750 ml salt solution. Sodium EGTA was added to the salt solution to the desired concentration. The salt solution in each bottle was kept at a constant ionic strength of 265 mM by addition of NaCl after pH adjustment. In one series without EGTA, NaCl was supplied to a final concentration of 550 mM. Granular sludge was washed with the salt solution and approximately 5 g dry weight was added to each serum vial. Closed vials were incubated overnight at 35°C in an end-over-end mixer (12 rpm).
When methanogenic activity of the sludge was measured too, EGTA incubations were performed in 120-ml serum vials with 50 ml medium (composition see above) under an atmosphere of N_2/CO_2 (4/1). After 18 h incubation with EGTA, substrate was added to a concentration of 40 mM.

Granule size and stability. The median diameter of the granules was used to characterize the size distribution. The sizes were quantified by image analysis as described previously (Grotenhuis et al. 1988).
The resistance of granular sludge against compression was chosen as a measure of granule strength (Hulshoff Pol et al. 1986). Granules (10 ml) were placed in a vertical cylinder (diameter 1.33 cm), a piston was moved downwards at constant speed (5 mm/min) and the resistance to compression was recorded with time by use of a tension and compression apparatus (Overload Dynamics S900, Overload Dynamics, Schiedam, The Netherlands). The resistance to compression sharply increased just before the granules disintegrated. Thereafter, the resistance to compression increased slowly. The pressure at which the transition of the two

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resistances occurred was taken as a measure of granule strength.

Physical disintegration of granular sludge. For physical disintegration granules (10 ml) were mixed with 30 ml medium and small aggregates were made by pressing the granules through a syringe (10 ml, Becton Dickinson, Etten-Leur, The Netherlands) without a needle. The suspension was then disintegrated further in a potter tube (Tamson, Zoetermeer, The Netherlands).

Activity measurement. The maximum methanogenic activity of native granules and of granules treated by EGTA or by physical disintegration was measured. The activity measurements were performed as described previously (Grotenhuis et al. 1991a). For quantification, gas samples (0.1 ml) were taken from the headspace at various periods of time and analysed. The activity on acetate was quantified by measuring the disappearance of acetate. All activity measurements were done in triplicate.

Analytical methods. The calcium, magnesium and phosphorus content of the granules was measured after wet oxidation with concentrated H_2SO_4 and H_2O_2 (Novozamsky et al. 1983). Calcium and magnesium were determined by atomic absorption spectrometry and phosphorus was quantified colorimetrically (van Schouwenburg and Walinga 1967).

Protein was measured according to Lowry et al. (1951) with bovine serum albumin as standard. All samples were first treated with 2 M HCl to remove sulphide and subsequently neutralized with 2 M NaOH.

The polysaccharide content was measured with the anthrone-sulphuric acid method (Dische 1962).

Methane was analysed by a Packard-Becker (Middelburg, The Netherlands) 406 gas chromatograph equipped with a thermal conductivity detector and molecular sieve column. Fatty acids were determined gas chromatographically after addition of Amberlite IR-120 (strong acid cation exchanger H^+-form) with a Chromosorb 101 (80 - 100 mesh) column 2 m x 2 mm; temperatures (°C): column, 150; injection port, 220; flame ionisation detector, 240; carrier gas (30 ml/min): nitrogen saturated with formic acid.

The dry weight of the granules was determined after drying overnight at 105°C. The percentage ash was measured after reheating for 2 h at 600°C. The organic material was calculated by subtraction of the ash weight from the dry weight. The initial dry weight was used for calculations.

Crystalline inorganic precipitates were analysed in dried granular sludge samples by roentgen diffraction with a Guynier camera (Cullity 1956, Anonymous 1987).
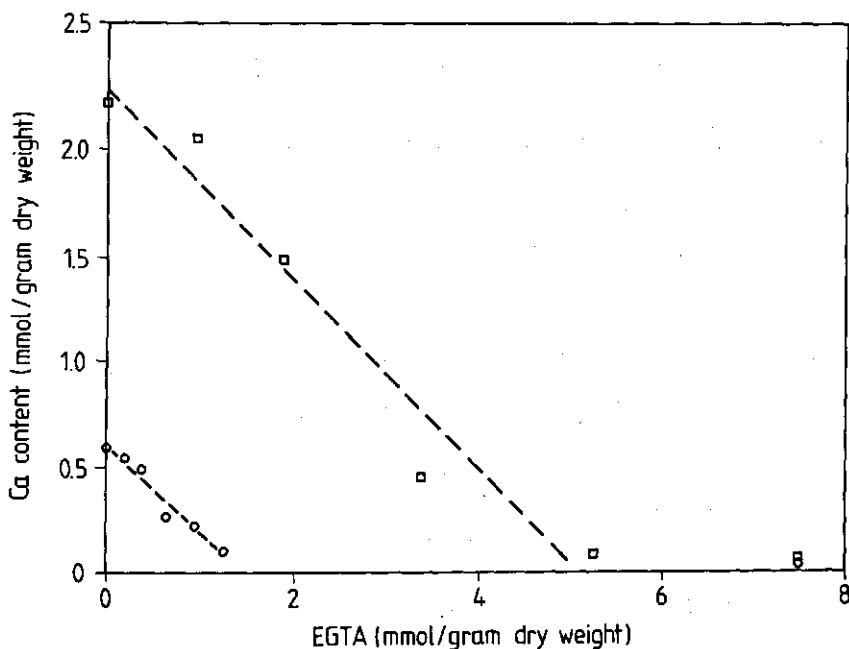
FIG. 1. Calcium content of propionate-grown methanogenic granular sludge (□) and of methanogenic granular sludge from a full scale upflow anaerobic sludge-blanket reactor of a paper-mill (O) as a function of the amount of ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid (EGTA) added. Correlation coefficients for calcium removal of the two sludges were 0.952 and 0.955, respectively

RESULTS

Effect of EGTA treatment on propionate-grown granular sludge

The calcium content of propionate-grown granules decreased linearly with the added amount of EGTA (Fig. 1). At calcium contents higher than 0.1 mmol/g dry weight, 2.1 mol EGTA was necessary to remove 1 mol calcium. This is more than twice the theoretical binding ratio of EGTA for calcium (Blaedel and Meloche 1963). The removal of calcium from the granules increased for 17 h during EGTA treatment. Thereafter, the calcium concentration in solution remained constant. Consequently, granules were exposed for 18 h to EGTA before further analysis were done. The magnesium content of the granular sludge was low (0.06 mmol/g dry weight) and did not change during incubation.

The phosphorus content of the granules decreased linearly with calcium (Table 1). Linear regression showed a molar P/Ca ratio of 0.59, which was very close to the P/Ca ratio of hydroxyapatite, $Ca_5OH(PO_4)_3$, a compound which was detected in propionate-grown granules by roentgen diffraction analysis (Fig. 2). The formation of hydroxyapatite in propionate-grown granules was predicted from the composition of

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TABLE 1. Effect of ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid EGTA treatment on propionate-grown granular methanogenic sludge[a]

EGTA added	Granule content		Release in medium[b]	
	Ca	P	Polysaccharide	Protein
(mmol/g dry weight)	(mmol/g dry weight)		(mg/g dry weight)	
0	2.22	1.45	0.94	< 1
0.94	2.05	1.57	0.88	1.5
1.88	1.48	1.13	1.04	3.0
3.38	0.45	0.49	1.23	10.1
5.25	0.09	0.31	1.23	10.2
7.50	0.06	0.29	1.33	89.8

[a] Ash content 23.5 %; biomass density 66.4 mg dry weight/ml
[b] After treatment for 18 h

the medium (Kissel et al. 1988). In granules incubated at 5.25 and 7.50 mmol EGTA/g dry weight all crystalline precipitates had been removed (data not shown).

The removal of calcium by EGTA caused disintegration of the granules (Fig. 3). The median granule diameter, which was taken as a measure of disintegration, correlated well with the concentration of EGTA (Fig. 4). The granule strength of propionate-grown granular sludge incubated without EGTA was $2.1.10^5$ N/m^2. Because of the disintegration of these granules after EGTA treatment, strength measurements were not meaningful.

At low EGTA/g dry weight ratios polysaccharides and protein were released proportionally to the amount of added EGTA. If propionate-grown sludge was treated with 5.25 mmol EGTA/g dry weight, 1.23 mg polysaccharides/g dry weight and 10.2 mg protein/g dry weight were released. After physical disintegration with negligible cell lysis of propionate-grown granular sludge 1.11 mg polysaccharides/g dry weight and 3.6 mg protein/g dry weight were found as extracellular polymers (Grotenhuis et al. 1991b). At 7.50 mmol EGTA/g dry weight the protein release increased rapidly in the medium (Table 1). This is an indication of cell lysis.

Effect of EGTA treatment on granular sludge from a paper-mill

EGTA treatment of full scale UASB granular sludge from a paper-mill showed a similar effect, with respect to the release of calcium, phosphorus, polysaccharide and protein, as that found with propionate-grown granules (Fig. 1, Table 2). The P/Ca ratio of 0.28 could not be assigned to a particular crystalline precipitate. Roentgen diffraction in untreated granules revealed only crystalline precipitates of $CaCO_3$ and
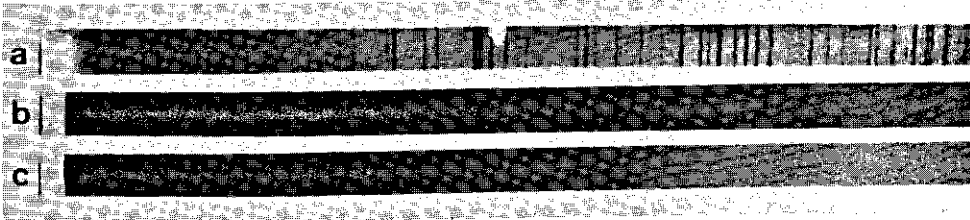
FIG. 2a-c. Roentgen diffraction patterns of inorganic precipitates in granular methanogenic sludge. a Calcium hydroxyapatite standard. b Propionate-grown sludge treated with 0.94 mmol EGTA/g dry weight. c Propionate-grown sludge treated with 7.5 mmol EGTA/g dry weight
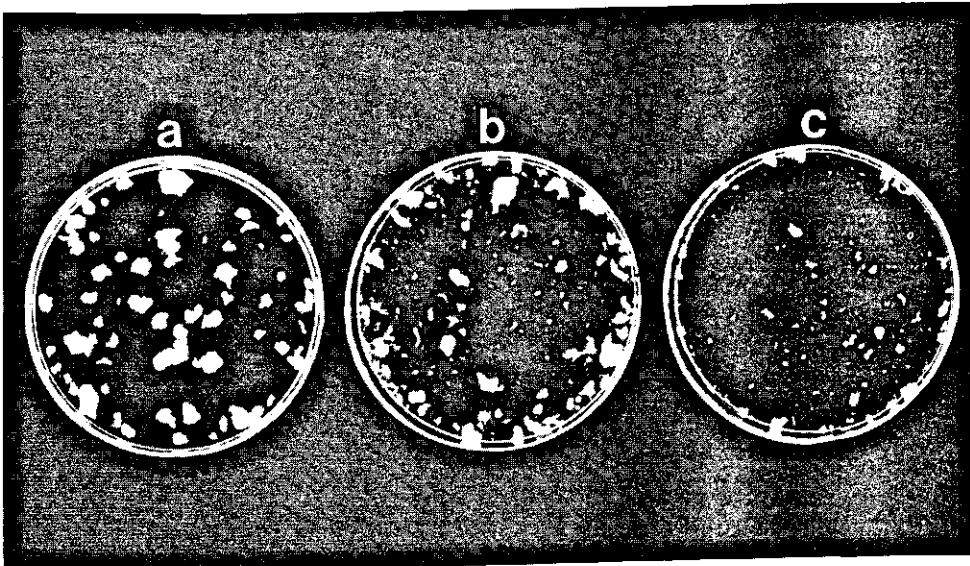
FIG. 3. Propionate-grown granular sludge after treatment at 0.00 (a), 3.38 (b) and 7.50 mmol EGTA/g dry weight (c)

kaolinite [AlSi$_4$O$_{10}$(OH)$_8$] (results not shown). After incubation with 7.50 mmol EGTA/g dry weight, only kaolinite was present.

In paper-mill granular sludge the amounts of extracted polymers reached levels of 5.63 mg polysaccharide/g dry weight and 81.8 mg protein/g dry weight after treatment with 7.5 mmol EGTA/g dry weight (Table 2). In contrast to the propionate-grown granules these granules did not disintegrate after EGTA treatment. However, calcium removal led to a decrease in granule strength (Table 2).

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TABLE 2. Effect of EGTA on granular methanogenic sludge grown in a full scale upflow anaerobic sludge-blanket reactor on paper-mill waste-water[a]

EGTA added	Granule content		Release into medium		Granule stability	
	Ca	P	Polysaccharide	Protein	Strength	Median diameter
(mmol/g dry weight)	(mmol/g dry weight)		(mg/g dry weight)		($N/m^2.10^5$)	(mm)
0	0.59	0.50	1.07	2.1	6.5	1.71
0.19	0.54	0.48	1.18	3.6	5.3	1.75
0.38	0.49	0.47	1.77	11.7	5.6	n.d.[b]
0.63	0.26	0.44	2.67	33.0	4.4	n.d.
0.94	0.22	0.42	3.81	54.2	4.2	n.d.
1.25	0.1	0.38	4.21	68.3	3.3	n.d.
7.5	0.04	0.31	5.63	81.8	2.7	1.80

[a] Ash content 15.7%; biomass density 100 mg dry weight/ml
[b] Not determineddisintegrated granules.

TABLE 3. Effect of EGTA treatment on the methanogenic activity of propionate-grown granular sludge

Substrate	Treatment	Activity (μmol substrate/g organic material/min)	Activity (%)
Propionate	Control[a]	10.7 ± 1.2	100
	EGTA (0.23 ± 0.01)[b]	11.2 ± 0.1	105
	EGTA (3.42 ± 0.14)	1.3 ± 0.5	12
Acetate	Control	10.7 ± 1.3	100
	EGTA (0.60 ± 0.04)	11.6 ± 1.8	108
	EGTA (5.75 ± 0.60)	7.2 ± 0.3	67

[a] No EGTA or NaCl added
[b] Values in parentheses represent the ratio of mmol EGTA/g dry weight

Activity of propionate-grown granules.

The effect of EGTA treatment on the maximum methanogenic activity of propionate-grown granular sludge was measured and compared with the activity of physically disintegrated sludge. EGTA treatment (3.42 mmol EGTA/g dry weight) of propionate-grown granular sludge led to a methanogenic activity decrease of 88% with propionate as test substrate. With acetate as test substrate the methanogenic activity was reduced only by 33% with 5.75 mmol EGTA/g dry weight (Table 3). At low EGTA concentrations propionate and acetate conversion was even slightly stimulated. An equal reduction of the acetoclastic methanogenic activity with high EGTA concentrations was found when only NaCl was added to the incubation medium at a similar ionic strength. In a separate experiment the effect of physical disintegration was determined. Here the propionate conversion decreased by 55%, whereas acetate conversion decreased only by 37%.
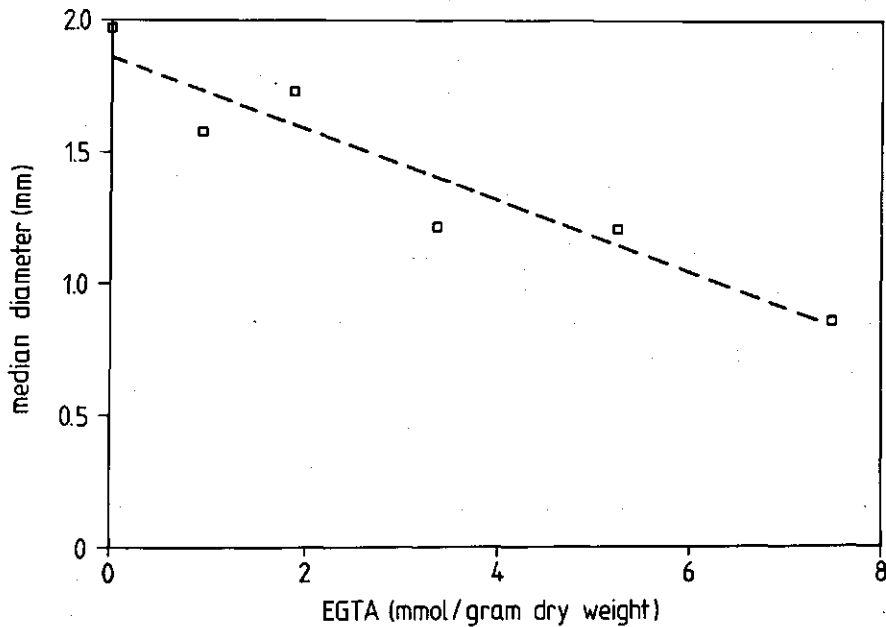
FIG. 4. Change in median diameter of the particles of propionate-grown methanogenic granular sludge as a function of the amount of EGTA

DISCUSSION

The stabilizing effect of calcium in propionate-grown granular sludge is most likely caused by the attachment of bacteria onto hydroxyapatite, leading to the formation of conglomerates of precipitate and immobilized cells. These inorganic precipitates are most probably the origin of the high granule strength of $2.1.10^5$ N/m^2, which was a factor 3-10 higher than the maximum gel strength measured for extracellular polysaccharides (Kang et al. 1982, Zevenhuizen 1984).

During EGTA treatment of inorganic precipitates of both sludge types, the EGTA/calcium ratio was more than twice the theoretical binding factor of EGTA for calcium (Blaedel and Meloche 1963). At the same time polysaccharides and proteins were extracted from the granular sludge. The polymers extracted by EGTA are most probably extracellular polymers of the bacteria in granular sludge. The increase in extracted polysaccharides and protein indicates that inorganic precipitates and extracellular polymers are both involved in the stabilization of methanogenic granules.

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The removal of polymers after EGTA treatment may be an indirect effect of the complexation of calcium by EGTA. A direct interaction of EGTA with lipopolysaccharides of the bacteria in the sludge granules may occur. After treatment of coliform bacteria with a similar chelant (EDTA), up to 20% (by weight) of their outer membrane was removed (Bayer and Leive 1977). Such direct interactions between extracellular polymers and EGTA may explain the higher EGTA/Ca ratio than theoretically expected.

EGTA at low concentrations had little effect on the methanogenic activity of the bacteria. However, at high EGTA concentration, the propionate conversion rate dropped by 88%. This marked decrease may be due to disintegration of the granules, since a physical disintegration of propionate-grown granular sludge caused a similar effect. A disruption of the specific spatial orientation of the hydrogen-producing and hydrogen-consuming bacteria will lead to an increase in the average distance between these organisms. Such an increase in the diffusion path for hydrogen between these two types of bacteria reduces the efficiency of hydrogen transfer and, as a consequence, the rate of propionate oxidation decreases (Grotenhuis et al. 1990, Stams et al. 1989). The acetoclastic activity, which is not dependent upon a bacteriological structure, was less affected. Dubourguier et al. (1988) observed similar effects for propionate and acetate conversion after physical granule disintegration.

This study shows that calcium is an essential element for the stabilization of granular methanogenic sludge, as indicated by the reduction in granule strength or the complete disintegration of granules after removal of calcium by EGTA.

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Chapter 5

BACTERIOLOGICAL COMPOSITION AND STRUCTURE OF GRANULAR SLUDGE ADAPTED TO DIFFERENT SUBSTRATES

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ABSTRACT

The bacteriological composition and ultrastructure of mesophilic granular methanogenic sludge from a large-scale Upflow Anaerobic Sludge Blanket (UASB) reactor treating wastewater from a sugar plant and of sludge granules adapted to ethanol and propionate were studied by counting different bacterial groups and by immuno-cytochemical methods. Propionate-grown granular sludge consisted of two types of clusters, those of a rod-shaped bacterium immunologically related to *Methanothrix soehngenii* and clusters consisting of two different types of bacteria with a specific spatial orientation. One of these bacteria reacted with antiserum against *Methanobrevibacter arboriphilus* AZ, whereas the other is most likely a propionate-oxidizing bacterium, immunologically unrelated to *Syntrophobacter wolinii*. Sludge granules obtained from a large-scale UASB reactor and granules cultivated on ethanol did not show such typical spacial orientation of bacteria.
Examination of the bacterial composition of the three types of granules by light and

electron microscopy, Most-Probable-Number countings and isolations showed that *Methanobrevibacter arboriphilus* and *Methanothrix soehngenii* were the most abundant hydrogenotrophic and acetoclastic methanogens in propionate-grown sludge. *Methanospirillum hungatei* and *Methanosarcina barkeri* predominated in ethanol-grown granules, whereas many morphotypes of methanogens were abundantly presentin granules from the full-scale reactor.

INTRODUCTION

Anaerobic oxidation of alcohols and fatty acids coupled to proton reduction can only proceed at low hydrogen partial pressures. For ethanol conversion a hydrogen partial pressure of less than 10^{-1} atm is required, whereas propionate degradation is possible solely below 10^{-4} atm (4, 13, 22). Such a low hydrogen partial pressure in methanogenic systems can only be realized by interspecies transfer of molecular hydrogen from obligately proton-reducing or facultative fermenting bacteria to hydrogen-oxidizing methanogens. A kinetic study with ethanol- and propionate-fed anaerobic continuously stirred tank reactors with a retention time of 15 days showed that an elevated hydrogen partial pressure caused by a perturbation with ethanol led to an accumulation of propionate (28). After 24 h the propionate conversion resumed when the hydrogen partial pressure had decreased again.

Besides hydrogen transfer also formate transfer may play a role in syntrophic oxidation of alcohols and fatty acids (2, 33, 35). Up to now it is unclear whether the process of interspecies electron transfer leads to a specific spatial orientation of bacteria of acetogenic bacteria and methanogenic bacteria. Such an orientation was suggested from kinetic and thermodynamic data (5, 10, 24, 34).

A nice model system to study a possible juxtaposition between bacteria is methanogenic granular sludge. In these densely packed biomass structures, microorganisms immobilize themselves. It can be assumed that immobilization is realized in places which are most optimal for the survival of the specific organisms. Methanogenic granules may be formed in upflow anaerobic sludge blanket (UASB) reactors (19). Two laboratory UASB reactors were inoculated with granular sludge from a liquid sugar plant and fed with either ethanol or propionate as sole carbon and energy source. The bacteriological shifts within the three granular sludge types were studied by using complementary techniques. The ultrastructure of ethanol- and propionate-grown granular sludge was studied with immunogold labeling techniques. Some preliminary results of this study have been presented before (11, 30).

MATERIALS AND METHODS

Bacteria and antisera. *Methanobrevibacter arboriphilus* AZ (DSM 744), *Methanospirillum hungatei* JF1 (DSM 864), *Pelobacter carbinolicus* (DSM 2909), *Methanosarcina barkeri* MS (DSM 800), *Methanosarcina mazei* MC3 (DSM 2907), *Methanobacterium thermoformicicum* Z245 (DSM 3720), *Methanobacterium thermoautotrophicum* Marburg (DSM 2133), *Methanobacterium thermoautotrophicum* delta H (DSM 1053) were obtained from the German Culture Collection DSM, Braunschweig. The cultures of *Methanothrix concilii* (DSM 3671), recently described as *Methanosaeta concilii* (23), *Methanothrix* FE (DSM 3013), and the cocultures of *Syntrophobacter wolinii* with *Desulfovibrio* G11 (DSM 2805) and *Syntrophomonas wolfei* with *Methanospirillum hungatei* JF1 (DSM 2245B) were kindly provided by Dr. H.C. Dubourguier, INRA, Villeneuve d'Ascq, France. *Methanothrix soehngenii* (DSM 2139) was the Opfikon strain described by Huser et al. (14). *Methanobacterium formicicum* D⁺ (9) was kindly provided by Dr. G.D. Vogels, Department of Microbiology, Catholic University of Nijmegen, The Netherlands. Strain EE121, a homoacetogenic ethanol degrading bacterium, was isolated from ethanol-grown granular sludge at our laboratory.

Antisera against whole cells from five bacterial strains were obtained by the immunization of rabbits, except the antiserum of the coculture *Syntrophobacter wolinii* and *Desulfovibrio* G11 which was kindly provided by Dr. H.C. Dubourguier, INRA, Villeneuve d'Ascq, France.

Media and cultivation. The growth medium contained (in g per l of demineralized water): KH_2PO_4, 0.41; $Na_2HPO_4.2H_2O$, 0.53; NH_4Cl, 0.3; NaCl, 0.3; $CaCl_2.2H_2O$, 0.11; $MgCl_2.6H_2O$, 0.1; $NaHCO_3$, 4; resazurin, 0.0005; cysteine HCl, 0.5; Na_2S, 0.24; vitamins 1 ml/l and trace elements 1 ml/l. Vitamins solution and trace elements solution were prepared as described before (40, 42). Sterile anaerobic techniques were followed as described by Balch et al. (1). Substrates were added by syringe from sterile stock solutions to give a final concentration of 20 mM. All organic acids were added as their sodium salts. For growth of hydrogenotrophic bacteria, the N_2/CO_2 (4/1) atmosphere was replaced by H_2/CO_2 (4/1). Both atmospheres were at 1.8 atm pressure. All incubations were performed in the dark at 37°C. The organisms were cultivated in 120-ml serum vials with butyl rubber stoppers in 40 ml of growth medium. Cell suspensions were centrifuged (10 min, 16,300 x g), washed three times with 0.01 M Tris-HCl, pH 7.2 and subsequently frozen at -20°C until further use.

Granular sludge types. Granular sludge was obtained from a 30 m³ UASB reactor at the Centrale Suiker Maatschappij (CSM) sugar refinery in Breda, The Netherlands. It had been cultivated at 35°C with wastewater of a liquid sugar plant, containing

sucrose, ethanol, lactate, acetate, propionate, butyrate and caproate at neutral pH (25). The sludge granules were stored anaerobically at 4°C until use.

Propionate-grown granular sludge and ethanol-grown granular sludge were cultivated in two 5-l UASB reactors at 35°C in the dark with mineral salt solution (12) and propionate or ethanol as the sole carbon and energy source. The reactors were inoculated with granular sludge from the UASB reactor at the CSM. The ethanol and propionate reactor were run for 6 and 36 months at a hydraulic retention time of 10 h and an influent concentration of 26 and 44 mM, respectively. Details of the operation of the UASB reactors were described before (12).

Activity measurements. Activity measurements were performed in 120-ml serum vials with 50 ml buffer solution as described previously (12). Liquid samples as well as gas samples were taken to analyze substrate removal and methane production.

Most Probable Number countings. Granular sludge (10 ml) was diluted with medium to 40 ml. Granules were gently homogenized and disintegrated with a 10-ml syringe without a needle by continuously taking up and ejecting the suspension followed by treatment with a Potter homogenizer (Tamson, Zoetermeer, The Netherlands). This disintegration procedure caused a negligible cell lysis as evidenced by the presence of low amounts of DNA in the culture medium (3). The various physiological cell types in the obtained suspension were enumerated by using the most probable number (MPN) technique (n=3) in media with different substrates. These tests were done in 35-ml glass tubes (Bellco Glass Inc., Vineland, N.J.) sealed with rubber stoppers and containing 9 ml of growth medium with 40 mM substrate and a gas phase of 1.8 atm N_2/CO_2 (4/1). For quantification of hydrogenotrophic bacteria, 1.8 atm H_2/CO_2 (4/1) was used as substrate. After 3 months of incubation at 37°C in the dark, methane formation and substrate depletion were determined by gas chromatography. The culture was examined by microscopy for microbial growth.

Preparation of antisera. Pure cultures of bacteria (40 ml) were centrifuged (20 min, 12,000 x g). The pellet was resuspended in 1 ml of phosphate-buffered saline (PBS, containing 1.0 mM KH_2PO_4, 3,4 mM K_2HPO_4, 37.3 mM NaCl at pH 7.2). Cells were inactivated by boiling for 10 min. Then, 1 ml of inactivated cells were injected subcutaneously into a rabbit together with 1 ml of Freund complete adjuvant (Sigma Chemical Co., St. Louis, Mo). After 28 days rabbits received a 1-ml booster together with 1 ml of Freund incomplete adjuvant (Sigma Chemical Co., St. Louis, Mo). 7 days later, 1.5 ml of blood was taken from an ear vein and the serological titer determined by an agglutination test. If the titer was not high enough, immunization was repeated weekly. After a high titer was present, rabbits were bled (40 ml) from the central ear artery. The blood was centrifuged (20 min, 5,000 x g) and serum

stored in aliquots of 1 ml at -20°C. To preserve the serum, 10 µl of 0.01% merthiolate solution was added to each aliquot.

Immunofluorescense. Bacterial samples (10 µl) were dried on micro-print slides and incubated with antiserum (1:10 dilution with PBS) for 30 min. The formed antigen-antibody complex was labeled with goat anti-rabbit fluorescein isothiocyanate (GAR-FITC, Sigma Chemical Co., St. Louis, Mo.). The reactions of antigens with an antiserum and the controls were performed on the same slide. Controls included the omission of the antiserum, or the omission of the goat anti-rabbit gamma globulin FITC conjugate. In these controls fluorescence was never observed. The slides were examined with an UV Leitz Dialux 20 EB microscope (Wild Leitz bv, Amsterdam, The Netherlands), using an I 2/3 filter block (6).

Electrophoresis and blotting. The specificity of the antisera was tested by Western-blots of protein patterns of several bacterial strains. Cell pellets were boiled with sample buffer according to Laemmli (18). After cleaning the crude extract with a 0.2 µm filter (Microgon Inc., Laguna Hills, Ca.), proteins were separated on a 10-15% sodium-dodecyl-sulfate-polyacrylamide-gel (Pharmacia Uppsala, Sweden). Subsequently proteins were transferred to a nitrocellulose membrane by electroblotting overnight at a constant voltage of 2 V. Blots were treated with 1% bovine serum albumin and incubated with antisera (1 h). Sera were visualized by binding of goat-anti-rabbit-gold (1:500, 10 nm) and amplified by silver staining with Intense BL (Janssen Chimica, Beerse, Belgium)

Antibody specificity. Specificity of antisera was tested with the following strains: *M. soehngenii* Opfikon, *M. concilii*, *Methanothrix* FE, *M. mazei* MC3, *M. arboriphilus* AZ, *M. hungatei* JF1, *P. carbinolicus*, strain EE121, *D.* G11, *M. formicicum* D$^+$, *M. thermoformicicum* Z245, *M. thermoautotrophicum* Marburg, and *M. thermoautotrophicum* delta H. The specificity of antisera was tested by gel-electroforesis in combination with Western-blots. The antiserum against EE121, an isolate from ethanol-grown granular sludge, showed some cross reaction with *P. carbinolicus*. The antiserum of *M. soehngenii*, showed only cross reaction with *M. concilii* and *Methanothrix* FE which confirmed the close relationship of these strains (36). With antiserum against *Methanothrix* FE similar results were found (38). *M. arboriphilus* AZ showed no cross reaction with other hydrogenotrophic methane bacteria.

Light Microscopy. Granules were squashed with the cover slip on a microscope slide for the estimation of the percentage of acetotrophic and hydrogenotrophic bacteria in methanogenic granules. A phase-contrast microscope was used for direct bacterial

counting and a Leitz Dialux 20 EB UV microscope (Wild Leitz bv, Amsterdam, The Netherlands) for quantification of hydrogenotrophic methanogens by autofluorescence.

Transmission electron microscopy. Granular sludge was fixed overnight at 4°C in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylatebuffer, pH 7.4. After washing in cacodylate buffer (3 times, 10 min) the granules were fixed in 1% OsO_4 in 0.1 M cacodylate buffer (2 h) and then dehydrated in a graded series of ethanol and propylene oxide. Granules were transferred to a propylene oxide-epon 812 mixture (10:1) and kept overnight at a relative humidity of 30% to permit the propylene oxide to evaporate slowly. Finally granules were transferred to fresh resin which was polymerized at 40°C for 16 h and at 80°C for 12 h. Ultrathin sections were cut with an ultramicrotome (Pharmacia LKB, Uppsala, Sweden) using glass knives, and collected on formvar-coated copper grids. Sections were poststained with uranyl acetate (30 min, 40°C) and lead citrate (40 s, 20°C).

For immunogold labeling, granules were fixed for 3 h in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH = 7.4). After 3 times of washing with cacodylate buffer, the granules were directly dehydrated in graded series of ethanol. Granules were embedded in London Resin White (Bio-Rad Laboratories, Richmond, Calif.). Copper grids with ultrathin sections were washed on a drop of 1% bovine serum albumin in PBS (pH 7.2, 3 x 10 min) and transferred to a drop of diluted antiserum (1:100 in PBS). After 1 h incubation at room temperature, the grids were washed with 1% bovine serum albumin in PBS (1 h) and then floated for 1 h on a goat-anti-rabbit-gold suspension (1:100, 10 nm, Janssen Chimica). Finally, grids were washed with 1% bovine serum albumin in PBS, PBS and milli Q water, for 1 h each. Poststaining of immunolabeled thin sections was done in uranylacetate (2% in H_2O) for 15 min at 20 °C. Sections were examined with a Philips EM 301 electron microscope at 60 kV.

Analytical methods. Methane was analyzed by a Packard-Becker 406 gas chromatograph (Packard Instrument Co., Inc., Rockville, Md.) with a thermal conductivity detector and molecular sieve, at 50°C. The carrier gas was argon at a flow rate of 20 ml/min. Fatty acids were determined after addition of amberlite IR-120 (strong acid cation exchanger H^+-form) by gas chromatography with a Chromosorb 101 (80 - 100 mesh) column 2 m x 2 mm; temperatures (°C): column 150, injection port 220, flame ionization detector 240; carrier gas (30 ml/min): nitrogen saturated with formic acid. Alcohols were determined with a Chrompack gas chromatograph 438 A with a sil5CB column (Chrompack, Middelburg, The Netherlands), 10 m x 0.53 mm; temperatures (°C): column 60, injectionport 250, flame ionisation detector 300; carrier gas (7.5 ml/min): nitrogen.

RESULTS

Bacterial determination and quantification. Using morphological and autofluorescence criteria in light microscopy, acetotrophic and hydrogenotrophic methanogenic bacteria in methanogenic granular sludge were distinguished and their percentages were determined. Acetotrophic bacteria resembling *Methanothrix* sp. and *Methanosarcina* sp. were found in CSM granular sludge, whereas only *Methanosarcina* sp. could be observed in ethanol-grown sludge and only *Methanothrix* sp. in propionate-grown sludge (Table 1). Hydrogenotrophic bacteria detected by autofluorescence were present in varying amounts in the three sludge types (Table 1).

Results of the MPN counts made of the three disintegrated granular methanogenic sludge types in anaerobic media with different carbon sources are given in Table 2. In the CSM sludge the metabolic group of glucose degraders is dominant over the methanogenic bacteria. The glucose degraders were reduced by a factor 10^4 after 6 months of granule growth on ethanol or after 36 months growth on propionate. A bacteriological shift to ethanol-degrading organisms in ethanol-grown granules was found. In propionate-grown granules the amount of propionate-degrading organisms was low. These granules showed a significant increase of hydrogenotrophic methanogenic and formate-degrading methanogenic bacteria compared to CSM and ethanol-grown granules. Compared with ethanol-grown granular sludge only low numbers of acetoclastic methanogens were counted in propionate-grown granules.

The highest dilutions where growth had occurred were tested for reaction with specific antisera (Table 3). In MPN tubes with acetate as substrate, specific immunological reaction was found with antiserum against both *M. barkeri* and *M. soehngenii* in CSM granules, whereas in ethanol and propionate-grown granular sludge only reaction with *M. barkeri* or *M. soehngenii* antiserum was detected, respectively (Table 3). The presence of *M. soehngenii* in propionate-grown sludge may explain the low numbers of acetotrophic methanogens in this sludge type compared with ethanol-grown granules. The numbers of *M. soehngenii* were underestimated in the test tubes used for the MPN counts at an incubation time of three months, since *Methanothrix* has a much lower growth rate than *M. barkeri* and *Methanothrix* grows in filaments which are not easily broken causing a detection limit in the MPN counts of not one cell but between 10 and 100 cells.

With the CSM granular sludge, labeling of the highest dilution of the tubes grown with H_2/CO_2 showed positive reaction with hydrogenotrophic methane bacteria *M. hungatei* JF1 and *M. arboriphilus* AZ. In ethanol- and propionate-grown sludge, only labeling with *M. hungatei* JF1 or *M. arboriphilus* AZ antiserum was found, respectively. Ethanol degrading bacteria reacting with the *P. carbinolicus* antiserum

TABLE 1. Relative numbers of acetotrophic and hydrogenotrophic methanogens in granular sludge counted directly under the light microscope.

	Relative mean percentage of methanogens that are[b]		
Sludge type[a]	Acetotrophs		Hydrogenotrophs
	Methanothrix spp.	*Methanosarcina* spp.	
	(%)	(%)	(%)
CSM	20	10	15
Ethanol	< 1	20	10
Propionate	30	< 1	40

[a] CSM is granular sludge grown on wastewater of a liquid sugar plant; Ethanol and Propionate are sludge types cultivated in 5 l UASB reactors with ethanol or propionate as sole carbon and energy source
[b] Acetotrophic methanogens were identified morphologically, and hydrogenotrophic methanogens by autofluorescence. It was assumed that each filament of *Methanothrix* spp. contained 1 cell per 3.3 μm of length (14). For *Methanosarcina* spp. each single cell was counted in the clusters. Each percentage value is the mean of at least 5 determinations, with a maximal standard deviation of 4 %. The percent composition of methanogens is based on the total number of cells

TABLE 2. Culture counts of different metabolic groups of bacteria present in three types of granular methanogenic sludge, with three tubes per dilution step.

	No. of bacteria/ml in the following sludge:		
Substrate	CSM	Ethanol	Propionate
Glucose	2.1×10^{12}	2.0×10^{8}	2.0×10^{8}
Ethanol	1.5×10^{9}	2.4×10^{10}	2.0×10^{8}
Propionate	2.4×10^{8}	3.0×10^{7}	4.6×10^{6}
H_2/CO_2	9.0×10^{10}	3.0×10^{9}	2.0×10^{12}
Formate	2.0×10^{9}	2.4×10^{8}	2.4×10^{11}
Acetate	2.4×10^{8}	9.0×10^{9}	4.6×10^{6}

TABLE 3. Indirect immunological detection of bacteria in the highest dilution of MPN measurements in three sludge types performed on five different substrates, by using FITC conjugated specific antibodies.

Antiserum against	Type of substrate used with the following sludge[a]		
	CSM	Ethanol	Propionate
Methanosarcina barkeri	1	1,5	None
Methanothrix soehngenii	1	None	1,5
Methanobrevibacter arboriphilus AZ	2	None	2
Methanospirillum hungatei JF1	2,3,5	2,3	None
Pelobacter carbinolicus	None	4	None

[a] Substrates tested: 1 = acetate, 2 = H_2/CO_2, 3 = formate, 4 = ethanol, 5 = propionate. In some cases, no FITC labeling was observed with any of the substrates (None).

were only present in ethanol tubes inoculated with ethanol-grown granular sludge. In none of the propionate-grown tubes labeling was found with antiserum of the coculture of *S. wolinii* and *Desulfovibrio* G11.

Substrate conversion rates. The bacteriological composition of granular methanogenic sludge was characterized by the use of activity measurements as described by Dolfing and Mulder (7). Table 4 shows potential methanogenic activities of CSM granular sludge and of granules grown on ethanol and propionate. For comparison, data of similar activity measurements done by Dolfing and Mulder (7) are included. These authors grew granules for only 3-4 months under defined conditions. Compared to the original granules the methanogenic activity of ethanol and propionate-grown granules increased for the test substrates ethanol and acetate, and propionate and acetate, respectively. Dolfing and Mulder (7) found a similar response but to a lower extent. Activity on propanol and butanol was high for ethanol-grown granules compared to propionate-grown granules, whereas isopropanol and butyrate were converted at much higher rate in propionate-grown granules.

Bacteriological structure. Transmission electron microscopy with ethanol-grown granular sludge showed sarcina-like structures and other bacteria without specific distribution (Fig. 1a).

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TABLE 4. Potential methanogenic activity on different substrates of granular sludge from a sugar refinery and from 5 l UASB reactors fed with ethanol or propionate

Substrate	Methanogenic activity of sludge types (μmol CH_4/g of VSS per min) in[a]:		
	CSM	Ethanol	Propionate
Ethanol	5.1 (7.1)[a]	36.8 (14.9)	2.3 (2.4)
Propionate	3.4 (2.8)	4.7 (0.04)	19.3 (5.6)
Acetate	3.3 (4.1)	28.2 (8.1)	20.5 (6.0)
Propanol		48.4	2.9
Isopropanol		0	17.3
Butanol		48.9	3.0
Butyrate	3.0	1.8	14.3

[a] Data in parenthesis from Dolfing and Mulder (7). These authors used CSM granular sludge grown on wastewater from a sugar factory; cultivated on ethanol or propionate in 1 l UASB reactors for 3-4 months. VSS, volatile suspended solids (in this case, the organic graction of the granules).

A clear structural organization was observed in propionate-grown granules, consisting of two different types of clusters (Fig. 1b). One cluster consisted of solely *Methanothrix* like bacteria (Fig. 1c), whereas the other consisted of two morphological types of bacteria (Fig. 1d) in which large spherical electron translucent bacteria are surrounded by small electron-opaque bacteria.

Immuno-electron microscopical identification. Immunogold labeling with antisera raised against EE121, *M. hungatei* JF1, and *M. mazei* MC3 showed specific labeling of randomly distributed bacteria in ethanol-grown granular sludge (results not shown).

In propionate-grown granular sludge the microcolony of *Methanothrix* like bacteria reacted specifically with the antiserum of *M. soehngenii* (Fig. 2a). In the microcolony type, consisting of two morphological different bacteria the dark stained bacteria labeled intensively with antiserum against *M. arboriphilus* AZ (Fig. 2b). Whereas the other, larger bacterium reacted weakly with the antisera tested. Even with the antiserum against *S. wolinii* only aspecific reaction was found, which indicates that the propionate-oxidizer in propionate-grown granular sludge is serologically not related to *S. wolinii*.
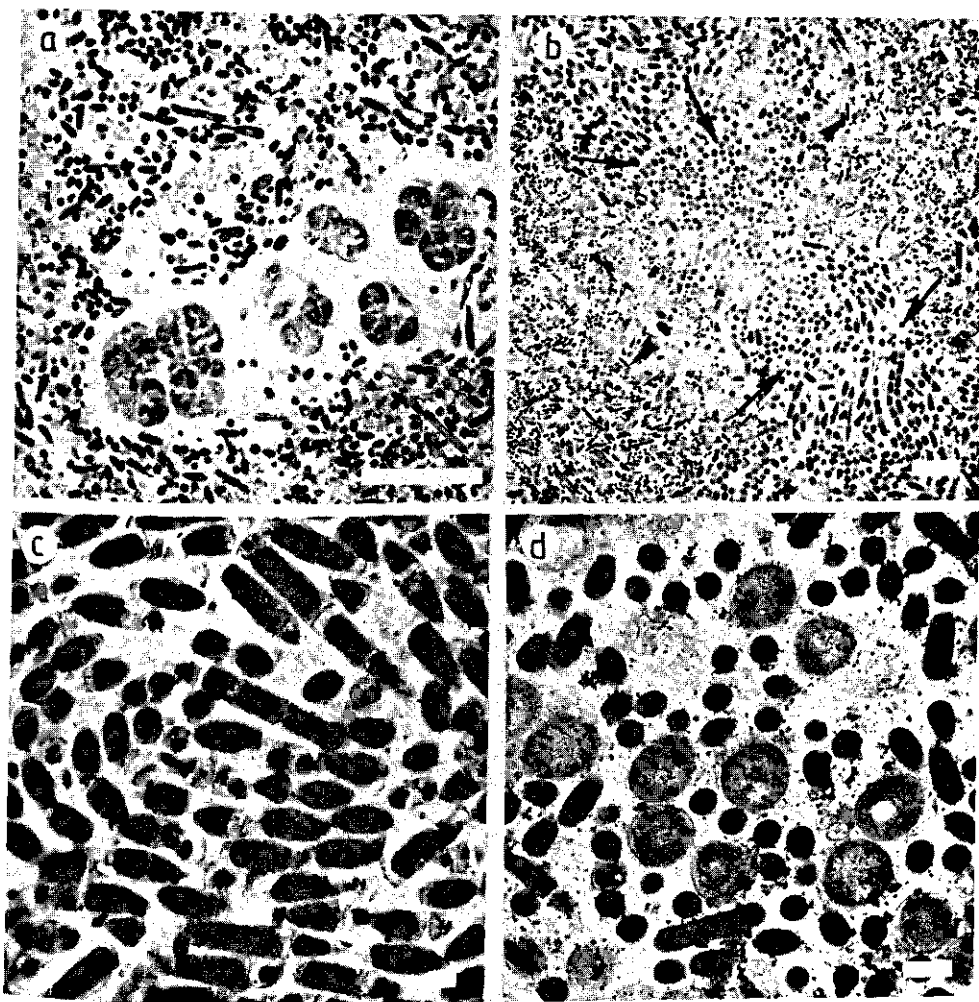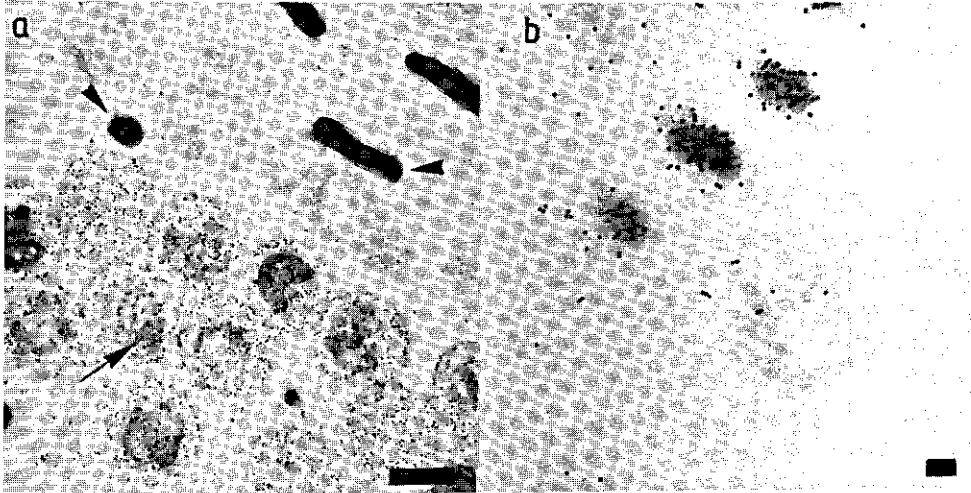
FIG. 1. Transmission electron micrographs of ultrathin sections of granular sludge. a. LR-White embedded section of ethanol-grown granular sludge showing *Methanosarcina* sp. and a variety of other bacteria (bar = 5 μm). b. Survey of two types of microcolonies observed in propionate-grown granular sludge, namely *Methanothrix* colonies (arrow) and a lattice type colony (arrow head), embedded in epon (bar = 5 μm). c. Detail of *Methanothrix* colony (bar = 0.5 μm). d. Detail of a lattice type colony (bar = 0.5 μm).

DISCUSSION

The shifts in both acetotrophic and hydrogenotrophic methanogens in granular sludge after adaptation to ethanol and propionate are remarkable. These population shifts can be explained by specific selection mechanisms. Propionate is only degraded if the hydrogen and acetate concentration are kept very low by the hydrogenotrophs and the acetate degraders (reaction 1, 3 and 4, Table 5) (36, 37). For ethanol oxidation hydrogen and acetate concentrations are less critical (reaction 2, Table 5) (31). In case ethanol is degraded faster than acetate a pH drop may occur. As a consequence ethanol conversion requires methanogens with a certain pH tolerance in the acidic range, whereas propionate conversion requires methanogens with a high hydrogen affinity ($\mu_{max}/2K_s$). *M. arboriphilus* strain AZ and *M. hungatei* strain JF1 nicely fulfil these requirements. Compared to *M. hungatei*, the affinity of *M. arboriphilus* for hydrogen is higher but the pH tolerance is lower (Table 6).

The shift in acetoclastic methanogens in the different types of sludge could be partly explained by different pH optima of *Methanosarcina* and *Methanothrix* (29, 42), but growth kinetic properties play a role as well. In the first-order region in a μ versus S plot, the bacterium with the highest ratio of $\mu_{max}/2K_s$ will win the competition. For acetate conversion by *Methanosarcina* and *Methanothrix* these ratios are in the same range. Therefore *Methanosarcina* and *Methanothrix* should grow equally well at low acetate concentrations (Table 6). *Methanosarcina*, however cannot convert acetate below a threshold of 0.2 mM, whereas the threshold for *Methanothrix* is 0.01 mM acetate. The differences in thresholds are due to different enzyme systems for the activation of acetate (15). If these threshold values are included in the μ versus S plot, it can be calculated that below the critical substrate concentration of 0.4 mM acetate *Methanothrix* will become dominant. Under optimal conditions μ_{max} for *Methanothrix* is 0.01 h^{-1} rather than 0.0032 h^{-1} (Jetten, personal communication, 1990). With this maximum growth rate *Methanothrix* would be able to outcompete *Methanosarcina* at acetate concentrations up to 1.76 mM.

The bacteriological shifts in the granular sludge found with the MPN countings were as expected, except for the low amount of propionate oxidizers in propionate-grown sludge. Recently, arguments were put forward that in suspended propionate oxidizing cultures formate transfer is relatively more important than hydrogen transfer (2, 33, 35). Based on the data presented here it can be postulated that in propionate-grown granules hydrogen transfer is more likely, because (i) the distance between propionate oxidizers and methanogens (< 0.5 μm, Fig. 1d) is short enough to allow hydrogen transfer (11, 30), and (ii) *M. arboriphilus*, a methanogen which is reported not to use formate (41), was found to be quite abundant in propionate-grown sludge. The conclusion that the hydrogen oxidizing methanogen in these structures is indeed *M. arboriphilus* is based on the following observations:

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FIG. 2. Transmission electron micrographs of ultrathin LR-White sections of a propionate-grown granule. a. Section labelled with antiserum against *Methanothrix soehngenii* conjugated with 10 nm goat-anti-rabbit-gold (bar = 0.5 µm). Note the specific labeling on *Methanothrix soehngenii* (arrow). *Methanobrevibacter arboriphilus* AZ (arrow head) and the unknown bacterium exhibiting only background labeling. b. Section labelled with an antiserum against *Methanobrevibacter arboriphilus* AZ conjugated with 10 nm goat-anti-rabbit-gold (bar = 0.1 µm). Note the dense labeling on *M. arboriphilus* AZ and the weak labeling on the unknown bacterium.

(i) Bacteria grown in the highest dilution on H_2/CO_2 in MPN tubes starting from propionate granules cross reacted exclusively with the antiserum against *M. arboriphilus* (Table 3). (ii) The highest dilutions were not able to use formate. They converted exclusively H_2/CO_2 to methane (not shown). (iii) The bacteria which reacted with the antiserum against *M. arboriphilus* showed in thin sections the typical morphology of this organism, namely straight rod with tapered ends. (iv) The study of Koornneef et al. (16) with the same sludge but another set of antibodies revealed *M. arboriphilus* as the dominant methanogen able to use exclusively H_2/CO_2 for methanogenesis.

When the fixed spatial orientation of bacteria was disrupted for MPN countings of propionate grown granules, *M. arboriphilus* might not be the proper methanogen to allow propionate oxidation in the suspension of the MPN tubes. The destruction of the specific spatial orientation, might have been less problematic in the other sludges, since *M. hungatei* was there abundantly present. In these cases, the electrons form propionate conversion could have been transported by formate as suggested by

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TABLE 5. Conversion of propionate and ethanol to methane in three steps

Reaction		$\Delta G°'$ [a] $kJ/reaction$
1 $CH_3CH_2COO^- + 3H_2O$	$\longrightarrow$ $CH_3COO^- + HCO_3^- + H^+ + 3H_2$	$+76.1$
2 $CH_3CH_2OH + H_2O$	$\longrightarrow$ $CH_3COO^- + H^+ + 2H_2$	$+9.6$
3 $CH_3COO^- + H_2O$	$\longrightarrow$ $CH_4 + HCO_3^-$	-31.0
4 $4H_2 + HCO_3^- + H^+$	$\longrightarrow$ $CH_4 + 3H_2O$	-135.6

[a] $\Delta G°'$ is the Gibbs free energy change under standard conditions at pH 7 (32).

TABLE 6. Monod growth kinetics and optimal pH of some acetotrophic and hydrogenotrophic methane bacteria.

Strain	Substrate	K_s (mM)	μ_{max} (h)$^{-1}$	$\mu_{max}/2K_s$ (mM.h)$^{-1}$	Optimum pH	Reference
Methanothrix soehngenii	acetate	0.7	0.0032	0.0023	7.4-7.8	14, 42
Methanosarcina 227	acetate	5	0.02-0.03	0.002-0.003	6.5-7	29, 21
Methanobrevibacter arboriphilus AZ	hydrogen	0.0066	0.144	10.9	7	41, 17
Methanospirillum hungatei JF1	hydrogen	0.005	0.05-0.06	5.0	6.6-7.4	26, 8
	formate	0.22	n.d.[a]	n.d.	n.d.	27

[a] n.d. means not determined

Boone et al. (2) and Thiele and Zeikus (33, 35). An alternative explanation for the low counts of propionate oxidizers in propionate-grown sludge may be that the most abundant propionate oxidizers are obligate proton reducing and thus not able to form formate.

The substrate conversion rates reflect the types and numbers of bacteria involved in substrate degradation. Two activities in propionate-grown sludge were remarkable, the high rate of butyrate and isopropanol degradation. Recently, it was found that propionate may partly be degraded via butyrate as an intermediate (36, 37). Our findings indicate that such a conversion might be more important than generally assumed. Immunological screening of the types of methanogens present in propionate-grown granules revealed the presence of *M. bryantii* (16). This bacterium was shown to oxidize isopropanol but not ethanol (4, 43).

MacLeod et al. (20) proposed a granule development model in which *Methanothrix* sp. positioned in the core of granular sludge would be surrounded by a second layer of H_2-producing acetogens and H_2-consuming bacteria. However, in propionate-grown sludge clusters of *Methanothrix* and clusters of *M. arboriphilus* together with the probable propionate oxidizing bacterium were found throughout the whole granule. Thus the bacteriological structure of propionate-grown granules did not confirm the layered structure predicted by MacLeod et al. (20).

Our results show clearly that the bacteriological composition of granular sludge shifts upon the type of growth substrate. With propionate as substrate not only a shift in bacterial composition but also a structural change took place; i.e. different bacteria formed structurally similar clusters within a granule. Propionate-grown granules are a nice example how thermodynamic and kinetic restrictions not only select for specific bacteria but also for the most optimal spatial orientation of the individual members of a bacterial substrate succession. The mechanical stability of the granules make them an ideal ecosystem for the study of microbial interactions, especially when the transfer of metabolites is involved. The forces which keep the spatial structure stable have as yet to be elucidated.

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Chapter 6

HYDROPHOBICITIES AND ELECTROPHORETIC MOBILITIES OF ANAEROBIC ISOLATES FROM METHANOGENIC GRANULAR SLUDGE

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ABSTRACT

The hydrophobicity and electrophoretic mobility of isolates from methanogenic anaerobic granular sludge were measured and compared with strains from culture collections. All new isolates were highly hydrophobic, indicating that the Upflow Anaerobic Sludge Blanket (UASB) reactor concept selects for hydrophobic bacteria. *Methanothrix soehngenii*, a methanogen often observed in methanogenic granular sludge, was highly hydrophobic and showed low electrophoretic mobility at pH 7. The role of this strain in the formation of methanogenic granular sludge is discussed.

Granular methanogenic sludge consists of a densely packed structure of anaerobic bacteria. Such microbial aggregates are preferentially formed in Upflow Anaerobic Sludge Blanket (UASB) reactors (9). The adhesion properties of bacteria present in high numbers in granular sludge adapted to ethanol or propionate were measured and compared with those of culture collection strains. Adhesion properties of cells play an important role in initial immobilization. Adhesion is strongly dependent on the surface characteristics of bacteria. Surface hydrophobicity and electrophoretic mobility may be taken as an indicator for the adhesion properties of bacteria (10, 11).

Methanobrevibacter arboriphilus (DSM 744), *Methanospirillum hungatei* (DSM 864), *Methanosarcina barkeri* (DSM 800), *Pelobacter carbinolicus* (DSM 2909), *Pelobacter carbinolicus* (DSM 2984), and *Pelobacter propionicus* (DSM 2379) were obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig. *Methanothrix soehngenii* was the Opfikon strain isolated by Huser et al. (6). Granular sludge originating from a 30 m^3 UASB reactor at the CSM sugar refinery in Breda, The Netherlands (14) was cultivated with ethanol or propionate as substrates under well defined conditions (3).

Isolation of bacteria was started by disintegration of methanogenic granules with a syringe without needle, followed by homogenization by means of a glass Potter homogenizer (Tamson, Zoetermeer, The Netherlands). Serial dilutions were made in triplicate in growth medium. Bottles were incubated for three months with ethanol, H_2/CO_2 or formate as sole carbon and energy source at 37°C. The highest dilution in which growth was observed was diluted in 2% agar media using the roll tube technique as described by Hungate (5). Pure cultures were obtained by repeating the roll tube procedure. Finally, single colonies were picked and transferred to liquid media. Purity was checked by microscopy, colony morphology and growth in complex media containing yeast extract and glucose. Cultivation of strains was routinely done in 120 ml vials containing 50 ml bicarbonate buffered medium and a gas phase of N_2/CO_2 (4/1) if acetate, ethanol or formate (20 mM) were the substrate or H_2/CO_2 (4/1) if H_2/CO_2 was the substrate. Both atmospheres were at 1.8 atm (182 kPa). Strain EE121 was isolated from methanogenic granules originating from an UASB-reactor that was fed ethanol as the sole carbon- and energy-source (15). Bacteria of this strain was Gram-positive, rod shaped with pointed ends and formed spores. They were between between 3.5 and 10 μm in length and about 1 μm in width. Cells moved by lateral flagellation. Antiserum raised against EE121 did not react with other isolated strains (PHC and EF strains). The following hydrogenotrophic methanogenic strains were isolated from propionate-grown granular sludge with H_2/CO_2 as substrate: PHC 252, PHC 254, PHC 256-1, PHC 256-2, PHC 259-1, PHC 259-3, PHC 2511. These strains produced methane from H_2/CO_2, were Gram-

negative, non motile, non-spore forming and showed autofluorescence at 420 nm. These bacteria were rod shaped 0.5 to 1 μm wide and 4 to 10 μm long. Antisera were prepared against strain PHC 252 and *Methanobrevibacter arboriphilus* AZ (DSM 744). Both antisera showed clear cross reactions with *M. arboriphilus* AZ (DSM 744) and all PHC strains indicating that the isolated strains belong to the genus *Methanobrevibacter*. From ethanol-grown granular sludge the isolates EF 173, EF 174, EF 175 were obtained with formate as the substrate. These strains produced acetate from formate, were Gram-positive, non motile, spore-forming and showed no autofluorescence at 420 nm. Cells were rod shaped, 1.5 μm wide and 6 to 9 μm long. Spores were 1.3 μm in width and 2 to 3 μm in length.

Bacterial hydrophobicity was quantified by the contact angle measurement method as described by van Loosdrecht et al. (10). Electrophoretic mobility was measured by laser Doppler velocimetry with a ZetaSizer (Malvern Instruments, Malvern, England) at pH 7 and pH 2 in 0.01 M phosphate-buffered saline (0.029 g KH_2PO_4, 0.119 g K_2PO_4, 0.493 g NaCl). In this procedure the migration velocity of bacteria in an electric field in an electrophoresis cell is determined with the Doppler-effect from a laser beam (11).

The hydrophobicity and electrophoretic mobility at two pH values were measured as indicators for the adhesion properties of the isolates. The obtained values were compared with some culture collection strains (Table 1). All isolates from granular sludge had a relative high contact angle and thus were rather hydrophobic especially if compared with contact angles of aerobic culture collection strains. Loosdrecht et al. (10) found for 20 aerobic strains contact angles between 20.1 and 44.7°. The PHC strains, which cross reacted with the antiserum of *M. arboriphilus* AZ, were more hydrophobic than the culture collection strain of *M. arboriphilus* AZ. Continuous growth of strain AZ in suspension and repeated transfers for many years before deposition at the German Type Culture Collection, DSM (19), may have selected for a less hydrophobic variety. Classical enrichment (batch and chemostat) and isolation techniques select for bacteria detaching easily from particles (soil, sediment, particular matter in water, etc.), growing as homogeneous suspensions and forming single colonies, preferably from single cells, on solid media. It is therefore not surprising that hydrophillic or highly charged hydrophobic bacteria are generally isolated with classical methods. For *Pelobacter propionicus*, isolated by these classical techniques from freshwater sediment (17), indeed a lower contact angle was found compared to the newly isolated anaerobic strains. In granular sludge a selection for well adhering (hydrophobic) bacteria has already taken place. The chance to isolate hydrophobic bacteria was enhanced in this study by directly isolating the most numerous bacteria from granular sludge after mechanically breaking up the granules. By following this strategy a counter selection for homogeneously growing, not

TABLE 1. Contact angles and electrophoretic mobility of several isolates from granular methanogenic sludge and anaerobic bacteria.

Bacterial strain	Contact angle[a] (°)	Electrophoretic mobility[b] (10^{-8} m.V^{-1}.s^{-1})	
		pH = 7	pH = 2
PHC 254	73.6	-2.14	-0.26
PHC 256-1	75.5	-1.65	-0.07
PHC 256-2	76.5	-2.30	-0.17
PHC 259-1	73.8	-2.33	-0.28
PHC 259-3	73.0	-2.04	0.11
PHC 2511	72.5	-2.12	1.01
EF 173	69.3	-2.51	0.32
EF 174	68.4	-1.92	0.51
EF 175	78.5	n.d.[c]	n.d.
EE121	82.0	-2.30	0.77
Methanobrevibacter arboriphilus AZ	53.8	-1.18	-0.19
Methanospirillum hungatei JF1	31.8	-2.19	0.29
Methanothrix soehngenii	69.4	-0.32	0.60
Methanosarcina barkeri	71.4	-2.40	n.d.
Pelobacter carbinolicus RE1	70.0	-2.88	0.81
Pelobacter carbinolicus BE4	86.1	-3.71	1.80
Pelobacter propionicus	49.0	-3.12	1.12

[a] Mean of 6 measurements, average standard deviation 1.4°
[b] Average standard deviation 0.15 x 10^{-8} m.V^{-1}.s^{-1}
[c] n.d. = not determined

adhering bacteria could be prevented. The high hydrophobicity of the two *Pelobacter carbinolicus* strains, isolated from granular sludge (2) is another indication for the selection of well adhering bacteria by this type of sludge.

The electrophoretic mobility of each of the anaerobic strains is different (Table 1). In wastewater with a relatively high ionic strength the charge of the bacteria is of less importance. Charge becomes a determining factor for adhesion only at ionic strength below 10 mM (12).

Methanothrix soehngenii, an acetoclastic methanogen, is often observed in large numbers in methanogenic granular sludge and methanogenic biofilms (1, 2, 4, 8, 13, 16). The other important acetoclastic methanogenic bacterium *Methanosarcina barkeri* has a much lower affinity for acetate than *M. soehngenii* (7) and is present at much lower numbers in methanogenic granular sludge of UASB reactors run for a long period at low acetate effluent concentrations (4). *M. soehngenii* is very hydrophobic and almost uncharged at pH 7 (Table 1). It also forms long intertwining threads in which other bacteria or inorganic precipitates are entangled. These growth kinetic properties, physicochemical properties and shape make *M. soehngenii* the ideal organism for adherence and give *Methanothrix* spp. a key role in stabilizing microbial agglomerates like granular sludge or biofilms (4, 18).

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Chapter 7

IMMOBILIZATION STUDY WITH A NEW HOMOACETOGENIC BACTERIUM *CLOSTRIDIUM GRANULARUM* SP NOV ISOLATED FROM ETHANOL-GROWN METHANOGENIC GRANULAR SLUDGE

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ABSTRACT

An ethanol degrading bacterium (EE121) was isolated from granular methanogenic sludge of an UASB reactor operated for 6 months with ethanol as sole substrate. The strain appeared to be a homoacetogenic bacterium immunologically related to *Clostridium aceticum* and *Clostridium formicoaceticum*, but it differed from these strains in the range of substrates used for growth. The new strain is described as the type strain of a new species, *Clostridium granularum* sp. nov. in the family of Bacterodaceae. *C. granularum* appeared to be highly hydrophobic, and was tested therefore for its ability to form aggregates. In batch cultures the strain grew as suspension, but in a recycle UASB reactor system it formed aggregates of 10 - 100 μm in diameter. In coculture with *Methanobrevibacter arboriphilus* strain AZ these aggregates were formed much faster than without the methanogen.

INTRODUCTION

In Upflow Anaerobic Sludge Bed (UASB) reactors used for wastewater treatment, bacteria are immobilized in densely packed bacterial aggregates of 1-3 mm in size (Lettinga et al. 1980). The formation of such granules was studied with complex wastewaters and mixtures of fatty acids using digested sewage sludge or cow manure as starting material (de Zeeuw 1984, Wiegant and Lettinga 1985, Hulshoff Pol 1989, Wu Weimin et al. 1987, Dolfing 1987). With different types of starting material, the granulation of biomass in UASB reactors with volatile fatty acids as feed was observed after 50-90 days (Hulshoff Pol 1989).

The mechanism of methanogenic granular sludge formation is not fully understood. In general, immobilization of bacteria into aggregates or biofilms is thought to be the net result of 1) transport of cells to the surface of an aggregate, 2) adsorption of cells, 3) growth and metabolic processes within biofilms and aggregates, and 4) detachment of cells from biofilms or aggregates (Characklis 1989). Initial immobilization of suspended bacteria by adhesion to solid surfaces is dependent on the charge and hydrophobicity of both the solid surface and the microbial cells. However, depending on the ionic strength of the medium charge was found to be of importance if the cell surface is hydrophilic (van Loosdrecht et al. 1987). Thusfar, the self-immobilization of anaerobic bacteria isolated from UASB systems was not yet investigated.

In this study an ethanol degrading anaerobic bacterium was isolated from granular sludge grown in a labscale UASB reactor fed with ethanol as sole carbon and energy source. The bacterium, which was present in the sludge in numbers of 10^{10} per ml of granules, appeared to be a new type of homoacetogenic bacterium. Preliminairy results of the characterization of the ethanol-degrading strain were presented before (Plugge et al., 1990). Here the strain is characterized further and tested for its ability to form aggregates from suspension in a special designed recycle UASB system. The strain formed in pure cultures aggregates of up to 100 μm in diameter. In the presence of a methanogen granule formation occurred even faster.

MATERIAL AND METHODS

Granular sludge. Starting material was obtained from a 30 m³ UASB reactor at the CSM sugar refinery in Breda, The Netherlands. This reactor had been operated as described by Pette and Versprille (1982). Ethanol-grown granular sludge was obtained by cultivating sludge from the sugar refinery in a 5 l UASB reactor at 35°C in the dark with ethanol (26 mM) as the sole carbon and energy source. The dilution rate was 0.1 h⁻¹, resulting in a liquid upflow velocity of 7 cm.h⁻¹. After inoculation,

the 5 l reactor was run for 6 months under the same conditions before isolation of bacteria was started. Further details of the 5 l UASB reactor were given previously (Grotenhuis et al. 1991).

Media and growth conditions. Cultivation of bacteria was routinely done in butyl rubber stoppered 120 ml vials containing 50 ml medium and a gas phase of N_2/CO_2 (4/1) or H_2/CO_2 (4/1) at a pressure of 1.8 atm. The mineral medium had the following composition (in g per l of demineralized water): KH_2PO_4, 0.41; $Na_2HPO_4.2H_2O$, 0.53; NH_4Cl, 0.3; NaCl, 0.3; $CaCl_2.2H_2O$, 0.11; $MgCl_2.6H_2O$, 0.1; $NaHCO_3$, 4; cystein HCl, 0.5; $Na_2S.9H_2O$, 0.24; resazurin, 0.0005. One liter medium also contained 1 ml vitamin solution (Wolin et al., 1963) and 1 ml trace elements solution (Zehnder et al., 1980). The medium was prepared as described by Houwen et al. (1987) and substrate (20 mM) was added aseptically from 1 or 2 M anaerobic stock solutions. Media for roll tubes and agar plates contained in addition 2 % agar (Difco). Agar plates were prepared in an anaerobic glovebox with a N_2/H_2 (96/4) gas phase. After inoculation, plates were incubated in jars with an atmosphere of N_2/CO_2 or H_2/CO_2 (4/1). For the determination of the pH optimum of the ethanol degrading bacterium phosphate (20 mM) instead of bicarbonate was used as buffer and the gas phase was N_2.

Isolation of strain EE121. Ethanol adapted granular sludge was disintegrated, using a syringe without needle and a glass Potter homogenizer (Tamson, Zoetermeer, The Netherlands). Serial dilutions of the suspension were made in triplicate in mineral medium with ethanol as the sole carbon and energy substrate. Bottles were incubated for three months at 37°C. The highest dilution in which growth was observed was used to incubate roll tubes (Hungate 1969). Single colonies were picked and purified further in roll tubes. Purity was checked by microscopical examination and colony morphology on agar plates.

Other bacteria. Methanobrevibacter arboriphilus AZ (DSM 744), *Pelobacter carbinolicus* (DSM 2909), *Pelobacter carbinolicus* (DSM 2984), and *Pelobacter propionicus* (DSM 2379) were obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany. *Clostridium formicoaceticum* (DSM 92) was a generous gift of Dr. J.R. Andreesen (University of Göttingen, Germany) and *C. magnum* (DSM 2767) of Dr. B. Schink (University of Konstanz, Germany). *C. aceticum* (DSM 1496) was from our own culture collection.

Electron microscopy. Samples for transmission electron microscopy were prepared by fixation overnight at 4°C in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. After washing in cacodylate buffer (3 times, 10 min) the

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samples were fixed for 2 h in 1% OsO_4 in 0.1 M cacodylate buffer, and then, dehydrated in graded series of ethanol and propylene oxide. Samples were embedded in Epon (A/B = 2/3) (Merck Nederland BV, Amsterdam, The Netherlands) and ultrathin sections were cut with an ultramicrotome (Pharmacia LKB, Uppsala, Sweden) using glass knives. Sections were collected on formvar coated copper grids and poststained with uranyl acetate (30 min., 40°C) and lead citrate (40 sec., 20°C).

For scanning electron microscopy, samples were concentrated on 0.2 μm nucleopore polycarbonate membrane filters. Filters were washed with 25 mM sodium cacodylate buffer (pH = 6.8) and fixed for 1 h in 2% glutaraldehyde in cacodylate buffer. After rinsing with cacodylate buffer a second fixation was carried out for 30 min. in 1% OsO_4 in cacodylate buffer. Dehydration was done in graded series of water-ethanol mixtures. Ethanol was replaced by liquid CO_2 and the samples were critical point dried (40°C, 100 atm.). Then, filters were attached to copper tape on a stub and sputter-coated with gold. Samples were examined at 15 kV in a JEOL 35C electron microscope.

Recycle UASB system. The recycle UASB system was constructed of a 1 l fermentor and a 0.5 l UASB reactor (h = 70 cm, ø = 3 cm) (Fig. 1). The fermenter top consisted of a 10 mm butylrubber plate. The UASB reactor was closed at the top by means of an inverted funnel (h = 7 cm, ø = 3 cm). To avoid dead volume at the bottom of the UASB, a glass tube with four side openings (ø = 0.1 cm) at a height of 1 cm from the bottom was used as influent point. A gas/liquid/solid separator (h = 10 cm, ø = 4.5 cm) was positioned on the UASB so that the effluent point was 5 cm below the top of the reactor. Sample ports at various heights of the UASB reactor were sealed with butylrubber stoppers. The fermentor was connected to the UASB by butylrubber tubing. The system operated anaerobically by continuous flushing with N_2/CO_2 (4/1) which was bubbled through a 1 mM Na_2S solution. The whole system was operated at an overpressure achieved by a 1 m water column. After inoculation (10 to 20%(v/v)) the system was operated in recycle mode. The suspension was pumped from the fermentor to the bottom of the UASB (ϕ_v = 50 ml/h) and recycled from the top of the UASB to the fermentor. New substrate (10-50 mM) was added in 200 ml fresh medium when the substrate had decreased below 4 mM. In case the substrate conversion rate was too low, presumably because of a high acetate concentration, 200 ml suspension was replaced by fresh medium without substrate. The UASB and the fermentor were uncoupled when approximately 3-4 ml of immobilized biomass was present at the bottom of the UASB reactor. The UASB was run further at a mean liquid retention time of 0.046 h^{-1}. Liquid samples from the UASB sample ports were taken by syringe anaerobically and aseptically. At the end of the experiment the immobilized biomass was collected, quantified and examined by electron microscopy.
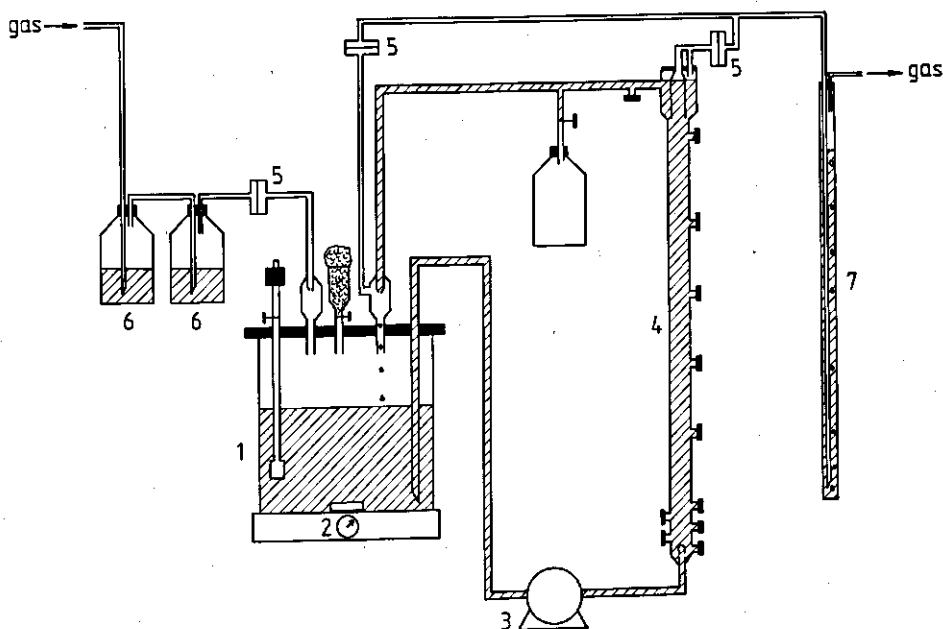
FIG. 1. Schematic diagram of the recycle UASB system. 1. fermentor; 2. Magenetic stirrer; 3. pump; 4. UASB reactor; 5. bacterial filter; 6. bottle with Na$_2$S; 7. 1 m water column.

Analytical methods. Fatty acids, ethanol, acetoin and 2,3-butanediol were determined by gas chromatography (Chrompack, Middelburg, The Netherlands) with a Chromosorb 101 column (Grotenhuis et al. 1991d). Gas samples were analysed for methane and hydrogen with a Packard 417 gaschromatograph equipped with a molecular sieve column (60-80 mesh) and a thermal conductive detector. The column was operated at 100°C and the carrier gas was argon at a flow rate of 20 ml.min^{-1}.

Glucose was determined with a glucose oxidase-test kit (Boehringer, Mannheim, Germany). Other sugars and organic acids were analysed by HPLC (Pharmacia LKB, Uppsala, Sweden) with a Biorad column. The column temperature was 55°C and the liquid phase 0.01 N H$_2$SO$_4$.

Calcium, phosphorous, dry weight and ash content were determined as described before (Grotenhuis et al., 1991a).

Miscellaneous methods. Flagella were stained according to the method of Mayfield and Inniss (1977) with a doubled HgCl$_2$ concentration. For determination of the G+C content of the DNA, frozen cells (3 g wet weight) were thawed and disrupted in a French pressure cell press at 10.000 psi. The DNA was purified by phenol extraction and hydroxyapatite adsorption according to Johnson (1981). The thermal denaturation procedure as described by Touzel et al. (1988) was used to determine

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TABLE 1. Growth yields and stoichiometry of ethanol (20 mM) and acetoin (11 mM) fermentation by strain EE121 in pure culture and in coculture with *Methanobrevibacter arboriphilus* AZ.[a]

Substrate	Substrate used	Products formed				Dry weight	Yield	Carbon recovery[b]
		Acetate	H$_2$	CH$_4$	Ethanol			
	(μmol)		(μmol)			(μg)	(mg/mmol)	(%)
Pure culture								
Ethanol	1093	1588	0.5			3990	3.7	115.7
Acetoin	555	1138	1		165	8514	15.3	116.3
Coculture								
Ethanol	955	1275	3.4	163		3717	4.0	117.0
Acetoin	640	1140	4	53	35	8280	12.9	92.7

[a] All data are the mean of at least three independent assays.
[b] Production and utilization of carbon dioxide was calculated according to the fermentation equations given by Thauer et al. (1977).

the G+C content.

Bacterial hydrophobicity was determined by contact angle measurements as described by van Loosdrecht et al. (1987).

Preparation of antisera and determination of immunofluorescence was done as described before (Grotenhuis et al. 1991d).

RESULTS

Isolation and general characteristics of strain EE121. In serial dilutions of disintegrated granular sludge adapted to ethanol for 6 months, growth with ethanol as substrate was found up to the 10^{10th} dilution. An ethanol-degrader (strain EE121) present in the highest dilution was isolated and characterized further. Strain EE121 was a Gram-positive rod shaped bacterium with slightly pointed ends. Its size was between 3.5 and 15 μm in length and about 1 μm in width. Cells were observed to be motile in log phase of glucose containing media only and peritrichous flagella were present. The cell wall architecture is clearly seen in thin sections (Fig. 2 a, c) which show the Gram-positive cell wall. The formation of holes in the cell wall upon incubation at 37°C in the absence of a fermentable substrate was regularly observed (Fig. 2 b). This process was often followed by total cell lysis. A rapid spore formation in the late log-phase was observed in glucose-containing media only. Elliptical spores (1 x 2.5 μm) were situated at the subterminal region of the cell. The DNA contains 28.3 mol% G+C. Isolate EE121 was highly hydrophobic as indicated by the contact angle of 82° ± 2.
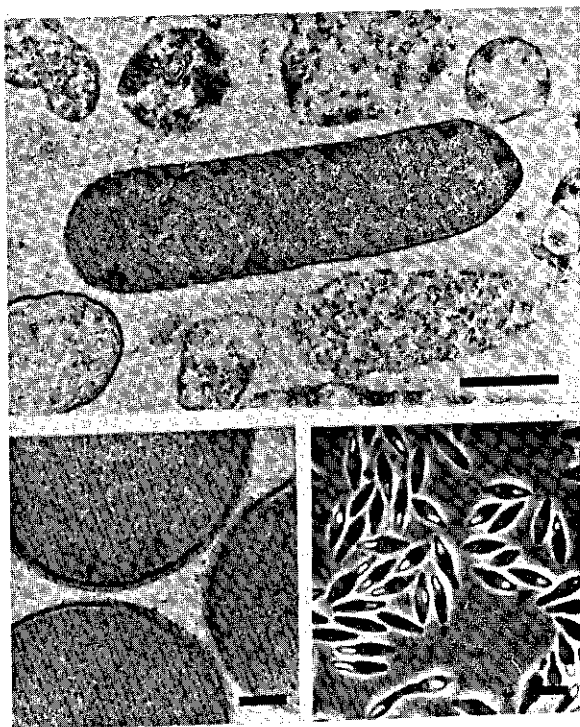
FIG. 2. a. Transmission electron micrograph of EE121. Note the interruptions in the cell wall. Bar = 1 μm. b. Transmission electron micrograph of EE121 showing the Gram-positive cell wall. Bar = 0.1 μm. c. Phase-contrast photomicrograph of EE121. Note the light spores in the subterminal region. Bar = 10 μm.

Fermentation of ethanol by strain EE121. With ethanol as the energy and carbon source the strain performed a homoacetogenic fermentation. About 1.5 mol of acetate was produced from 1 mol ethanol (Table 1). Less then 0.1% of the reducing equivalents formed in the oxidation of ethanol were disposed as hydrogen. The pH optimum was between 7 and 7.5 and the optimum temperature between 30 and 37°C. The μ_{max} on ethanol was 0.15 ± 0.02 h^{-1}. In the presence of the hydrogen consuming methanogen *Methanobrevibacter arboriphilus* AZ ethanol conversion was more rapid than in pure culture. In coculture at pH 7.2 and 35 °C, approximately 1 mol of acetate and 0.5 mol CH_4 was formed from 1 mol of ethanol. However, most often higher amounts of acetate and lower amounts of methane were produced (Table 1). Formate was never observed as intermediate or end product of ethanol fermentation, neither in pure nor in mixed culture experiments. Nitrate, sulfate, sulfur, thiosulfate or fumarate were not reduced, in the presence of ethanol.

TABLE 2. Substrates used by strain EE121 and by other mesophilic

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TABLE 2. Substrates used by strain EE121 and by other mesophilic homoacetogenic anaerobes and *Pelobacter carbinolicus*

Substrate	EE121[a]	Clostridium aceticum[b]	Clostridium formicoaceticum[c]	Acetobacterium woodii[d]	Pelobacter carbinolicus[e]	Clostridium magnum[f]
H2/CO2	+	+	-	+	-	+[g]
Formate	+	+	-	+	-	+[g]
Methanol	+	-	+	- (+ in k)	-	-
Ethylene glycol	-	+	-	+[j]	+	-
Ethanol	+	+	+	-	+[h]	-
2,3-Butanediol	+	+[e]	n.d.[m]	+[e,i]	+	+
Acetoin	+	+[e]	n.d.	+[e,i]	+	+
1-Propanol	+	n.d.	n.d.	-	+[h]	n.d.
2-Propanol	+	-	n.d.	-	-	n.d.
1-Butanol	+	n.d.	n.d.	-	+[h]	n.d.
2-Butanol	+	n.d.	n.d.	-	n.d.	n.d.
Fructose	+	+	+	+	-	+
Glucose	+	-	-	+	-	+
Sucrose	+	-	-	-	-	+
Galactose	+	-	-	-	n.d.	n.d.
Arabinose	-	n.d.	-	-	n.d.	-
Xylose	+	-	-	-	-	+
Ribose	-	+	+	-	n.d.	-
Glycerol	+	-	+	-	-	-
Glycerate	-	n.d.	n.d.	+	-	-
Fumarate	-	+	+	-	-	-
Citrate	+	-	-	-[*]	-	+
Succinate	-	-	-	-	-	-
Lactate	-	-	+	+	n.d.	-
Pyruvate	+	+	+	+[e]	n.d.	+[l]
Malate	+	+	+	-	-	+
Glutamate	-	+	+	-	n.d.	-
Aspartate	-	n.d.	n.d.	-	n.d.	n.d.
Serine	-	+	-	-	n.d.	-

[a] Less than 1 mM acetate formed from substrate indicated by -
[b] Data from Braun et al. 1981 and Adamse 1980
[c] Data from Andreesen et al. 1970
[d] Data from Balch et al. 1977
[e] Data from Schink 1984a
[f] Data from Schink 1984b
[g] Growth observed on H2/CO2 and formate if grown in presence of yeast extract (Bomar et al., 1991)
[h] Growth possible in mixed culture with *Acetobacterium woodii* or Methanospirillum hungatei (Schink, 1984a)
[i] Not all strains grow on this substrate (Schink, 1984b)
[j] After Schink and Stieb 1983
[k] Data from Bache and Pfennig 1981
[l] Weak response Schink 1984b
[m] n.d. means not determined

Fermentation of other substrates. The strain had a very broad substrate spectrum (Table 2). Besides C_1 substrates like methanol, formate, CO and H_2/CO_2 also sugars, organic acids and alcohols were utilized. With most of the substrates, acetate and small amounts of hydrogen were found as end products. During growth on acetoin also ethanol was measured in the medium. The growth rate on acetoin was 0.24 ± 0.08 h^{-1}. Growth on formate led to intermediate formation of high amounts of hydrogen in the gas phase. After 30 days of incubation at 37°C in a medium with 50 mmol formate as sole substrate about 30% had been converted to hydrogen and carbon dioxide.

Comparison with other ethanol-degrading anaerobes.
Indirect immunofluorescence by FITC labelling of antiserum raised against strain EE121 showed a positive reaction with *C. aceticum, C. formicoaceticum*, whereas no reaction occurred with *A. woodii* and *C. magnum*. Antiserum raised against *P. carbinolicus* resulted only in a slight positive reaction with EE121. Some morphological and physiological properties of EE121 and of other homoacetogenic bacteria and *P. carbinolicus* are listed in Table 3. Strain EE121 differed with all of them in at least two properties. Strain EE121 used a larger range of sugars than *C. aceticum, C. formicoaceticum, A. woodii* and *P. carbinolicus*. EE121 used the same sugars as *Clostridium magnum*, but this strain cannot use methanol and ethanol.

Immobilization studies. To investigate the immobilization capacity of suspended cells of strain EE121 into aggregates, recycle UASB systems were inoculated with 10% of batch cultures (about 10^8 cells/ml) grown on ethanol or acetoin. The last substrate was chosen since EE121 showed fast growth with acetoin in pure culture. In a coculture experiment, 10% inoculum (about 10^8 cells/ml) of EE121 grown on ethanol and 10% *Methanobrevibacter arboriphilus* AZ grown on hydrogen was used. The systems were operated in a recycle mode until 3-4 ml immobilized biomass was present at the bottom of the UASB reactor. After 114 days the pure culture grown on ethanol was run as UASB only, whereas the mixed culture and EE121 grown with acetoin had formed enough immobilized biomass for continuous operation after 45 and 64 days, respectively. After start of the continuous operation the substrate conversion rate increased, whereas due to continuous operation the acetate level dropped in the reactors.

Some data of the reactor performance and characteristics of the immobilized biomass at the end of the experiments are given in Table 4. The highest immobilization yield expressed as g dry biomass per mol acetate formed was found for the coculture. Aggregates which were formed in the UASB reactors reached sizes of up to 100 μm in diameter. They consisted of both microbial cells and inorganic

TABLE 3. Several properties of strain EE 121 and other mesophilic homoacetogenic anaerobes and *Pelobacter carbinolicus*

Property	EE121	*Clostridium aceticum*[a]	*Clostridium formicoaceticum*[b]	*Acetobacterium woodii*[c]	*Pelobacter carbinolicus*[d]	*Clostridium magnum*[e]
Length (μm)	3.5-15	5	8	2	3	4-16
Width (μm)	1	1	1.4	1	0.6	1-4
Spores	elliptical subterminal	round terminal	round terminal	-	-	elliptical central
Motility	+	+	+	+	-	+
Flagella	peritrichous	peritrichous	peritrichous	subterminal	-	polar
G+C (mol%)	28.3	33	n.d.	39	52.3	29.1
Gram-stain	+	-	-	+	-	-
Temp. opt. (°C)	30-37	30	37	30	35	30-32
pH optimum	7-7.5	8.3	7.8-7.2	5	6.5-7.2	7.0

[a] Data from Braun et al. 1981 and Adamse 1980
[b] Data from Andreesen et al. 1970
[c] Data from Balch et al. 1977
[d] Data from Schink 1984a
[e] Data from Schink 1984b

precipitates (Fig. 3). A large percentage of cells embedded in the inorganic precipitates were damaged in a similar fashion as observed in starved batch cultures. In aggregates of the coculture *M. arboriphilus* AZ was regularly observed as long thin strands (Fig 3b). This strain was described to be pleomorphic (Zehnder and Wuhrmann, 1977). Elongated cells are formed at low growth rates under nutrient limitation. The hydrophobicity of the aggregates was lower than the hydrophobicity of pure cultures. This may be due to the presence of inorganic material within the aggregates. Chemical analysis showed that the inorganic precipitates mainly consisted of calcium phosphate.
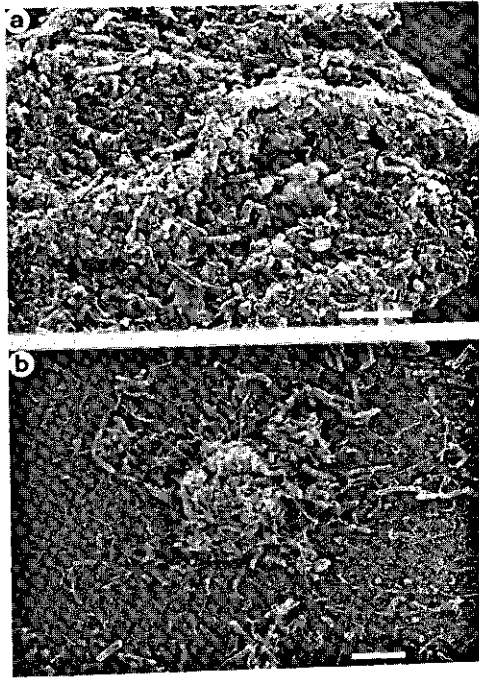
FIG. 3. Scanning electron micrographs of aggregates formed in recycle UASB system consisting of EE121 (a) and in coculture with *Methanobrevibacter arboriphilus* AZ (b). Bar = 10 μm.

TABLE 4. Comparison immobilization EE121 with ethanol, acetoin and in coculture with *Methanobrevibacter arboriphilus* AZ on ethanol in a recycle UASB system at the end of the experiments.

Substrate	Pure culture ethanol	Pure culture acetoin	Coculture ethanol
Operation time (d)	160	110	133
Loading rate (mmol/l.d)	12.3	15.0	39.2
Substrate removal (%)	99.0	90.7	87.8
Hydrophobicity of cells (°)	78.8	75.2	38.2
Immobilized biomass (mg)	71.7	78.3	203.1
Ash content (%)	10.5	9.9	19.3
Total substrate used (mmol)	394.0	324.1	1025.5
Immobilization yield (mg dry weight/mmol acetate formed)	0.182	0.121	0.198

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DISCUSSION

Classification of strain EE121. A Gram-positive homoacetogenic, sporeforming, strict anaerobic bacterium was the most numerous ethanol-degrading bacterium which we could isolate from ethanol-adapted granular sludge. The following properties indicate that strain EE121 is a member of the genus *Clostridium*: forming endospores, strictly anaerobic growth, absence of dissimilatory sulfate reduction, low G+C content, and positive Gram type of cell wall. Though antiserum against strain EE121 showed cross reaction with *C. aceticum* and *C. formicoaceticum*, our isolate differed in Gram reaction, in its capacity to use glucose, sucrose, galactose and xylose, and its inability to ferment ribose. Strain EE121 used the same sugars as *C. magnum* and had almost the same G+C content, but differed in properties like Gram reaction and use of alcohols. In view of the marked differences of strain EE121 with described homoacetogenic clostridia, a new species is proposed: *Clostridium granularum*.

C. granularum sp. nov., gra'nu.la.rum M.L. adj. *granulus* granule, referring to granular biomass from which the strain was isolated.

Rod-shaped cells, 3.5-15 μm in size with rounded ends. Motile, Gram-positive, elliptical spores formed subterminally.

Strictly anaerobic chemoorganotroph. H_2/CO_2, formate, methanol, ethanol, 2,3-butanediol, acetoin, 1-propanol, 2-propanol, 1-butanol, 2-butanol, fructose, glucose, sucrose, galactose, xylose, glycerol, citrate, pyruvate, and malate used for growth. No growth on ethylene glycol, arabinose, ribose, glycerate, fumarate, succinate, lactate, glutamate, aspartate, and serine. Nitrate, sulfate, sulfur, thiosulfate or fumarate not reduced.

Selective enrichment from ethanol-grown granular sludge in carbonate-buffered mineral medium with vitamins and ethanol as sole substrate.

pH range: 6.5-8.0, optimum 7.0-7.5.

Temperature range: 15°C-45°C, optimum temperature 30°C-37°C.

DNA base ratio: 28.3 ± 1.0% G + C (thermal denaturation).

Habitat: mesophilic anaerobic methanogenic granular sludge from UASB reactors.

Type strain: EE121, is deposited in Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

Immobilization of strain EE121. Initial immobilization of suspended cells of strain EE121 was studied in a special designed UASB-recycle system. This reactor system allows study of aggregate formation at a low biomass concentrations. Bacteria which are washed out from the UASB reactor are recycled in the system and obtain new possibilities for aggregation. Results showed immobilization and aggregate formation in the UASB reactor without dilution of the bacterial culture, whereas suspended

growth was only observed in batch cultures in serum bottles. Thus the prerequisite stated by Heijnen (1984) that biofilm formation only occurs at dilution rates, higher than the maximum specific growth rates of the bacteria is not essential for our strain in the recycle UASB system. High amounts of calcium phosphate precipitates were found in aggregates of strain EE121. This observation suggests that the formation of calcium phosphate precipitates plays an essential role in aggregate formation. The exact mechanism of such stimulation is not known, but the formation of calcium phosphate was found to be advantageous in the start-up of UASB reactors (Hulshoff Pol et al. 1983, Alibhai and Forster 1986, Mahoney et al. 1987). Calcium precipitates may serve as support material for the adhesion of bacteria (Arvin and Kristensen 1983, Harvey et al. 1984) and increase the aggregate stability (Grotenhuis et al 1988, Grotenhuis et al., 1991b).

In this study it was found that attached growth preferently occurred in a mixed bacterial consortium of hydrogen producing bacteria and hydrogenotrophic methanogens. Strain EE121 had good adhesion characteristics, as it was directly isolated as the most numerous ethanol degrading bacterium from granular sludge after mechanically breaking up the granules (Grotenhuis et al., 1991c). If isolation techniques are used which are directed to the isolation of attached bacteria (Szewzyk and Pfennig, 1986) or attached consortia of bacteria degrading one substrate, the isolation of strains with a high immobilization yield will be most probably even more successful.

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Chapter 8

ROLE OF SUBSTRATE CONCENTRATION IN PARTICLE SIZE DISTRIBUTION OF METHANOGENIC GRANULAR SLUDGE IN UASB REACTORS

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ABSTRACT

The effect of influent substrate concentration on particle size distribution of methanogenic granular sludge was studied in laboratory-scale UASB reactors fed with propionate as sole carbon and energy source. A mean biomass increase of 60.9 mg VSS/d and 0.95 ml sludge/d was measured in 5 UASB reactors operated in parallel mode, resulting in a mean SVI of 7.8 ml sludge/g dry wt. Particle size distributions were measured by a gravimetric method and by direct image analysis. Median granule diameter increased with increasing influent substrate concentration and decreased with decreasing concentration. Increasing the influent concentration led to an increase of the methanogenic activity of the granules with propionate or acetate as test substrate.

INTRODUCTION

In upflow anaerobic sludge blanket (UASB) reactors, a dense granular biomass with high solids retention time may be formed (Lettinga et al., 1980). Well developed granules are characterized by high settleability and high methanogenic activity. These properties permit use of a relatively small anaerobic system to provide an efficient treatment of large wastewater streams. Many full scale reactors have been built in the past decade and data on their performance are available (Lettinga et al., 1987; de Zeeuw, 1988). From these studies, it is clear that both the type of wastewater and the hydraulic properties of the reactor are important determinants of the formation and stability of granular methanogenic sludge. Hulshoff Pol et al. (1983) showed that in systems fed fatty acid mixtures, granulation occurred at organic loading rates higher than 0.6 kg COD/kg VSS.day. At such loadings, granules have also been easily obtained with wastewater from a sugar refinery, but were not formed with wastewater from a slaughterhouse (Sayed et al., 1984; Sayed et al., 1987). With the latter type of wastewater granules from a sugar refinery even disintegrated upon performance.

Granule formation may be initiated by bacterial adhesion to inorganic precipitates such as iron sulfide or calcium phosphate (Mahoney et al., 1987, Harvey et al., 1984) or by adhesion of bacteria to each other. In either case hydrophobicity and charge of the surfaces are important (Loosdrecht et al., 1987 (a, b)). Both also have an impact on granule stability. Alteration in surface charge by removal of calcium, for example, may lead to a decrease in granule strength and in some cases to complete disintegration of granules (Grotenhuis et al., 1988).

In addition to granule formation and stability, good settleability is required for satisfactory performance of UASB reactors. Settleability is dependent on density and size of the granules. Density of UASB granules is greatly influenced by the presence of inorganic precipitates and the inclusion of gas (Hulshoff Pol et al. 1986). The average particle size of granular sludge is dependent on multiple factors including nutrient supply, type of nutrients, granule growth rate, substrate limitation inside the granules, shear forces (Tramper et al., 1984), and production of extracellular biopolymers as well as gas generation and formation of inorganic precipitates. Particle size distribution may be considered as a characteristic parameter of granulated biomass.

In this study, well defined granules were grown with propionate as sole carbon and energy substrate and the effect of substrate concentration on particle size distribution was investigated.
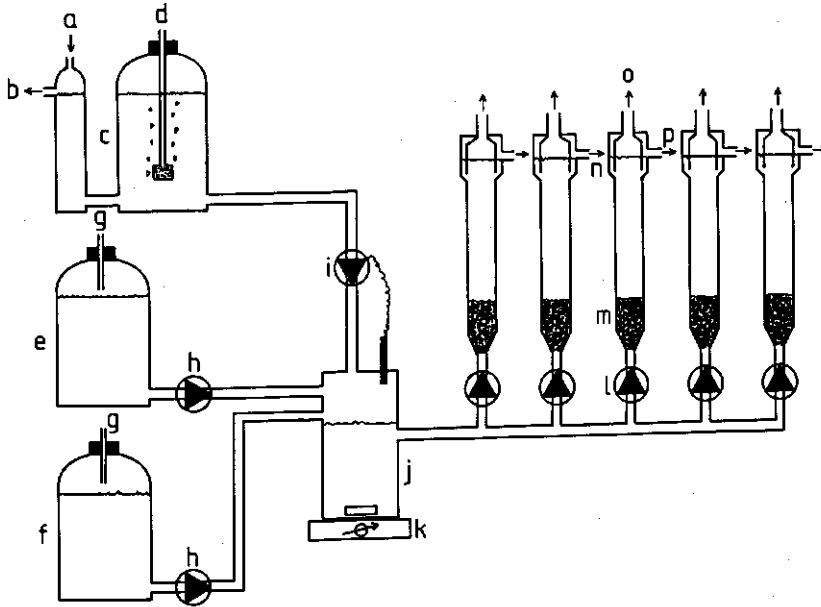
FIG. 1. Schematic diagram of the reactor system. (a) tap water inlet; (b) tap water outlet; (c) tap water reservoir; (d) gas flush N_2/CO_2 (4/1); (e) and (f) concentrated solutions; (g) connection to gas reservoir with N_2/CO_2 (4/1); (h) pump; (i) tap water pump with liquid level control; (j) mixing vessel; (k) magnetic stirrer; (l) pump; (m) granular sludge bed; (n) gas/liquid/solid separator; (o) connection to gas bag; (p) effluent outlet equipped with water seal.

MATERIAL AND METHODS

Experimental procedure

Five glass UASB reactors (h = 70 cm, ∅ = 3 cm, V = 0.5 l) were made according to the design of Hulshoff Pol *et al.* (1983) and Dolfing (1987) (Figure 1). To avoid dead volume at the bottom of the reactor, a glass tube (∅ = 0.8 cm) with four side openings (∅ = 0.1 cm) at a height of 1 cm from the bottom was used as the influent point.

A gas/liquid/solid separator (h = 10 cm, ∅ = 4.5 cm) was positioned on each reactor so that the effluent point was 5 cm below the top of the reactor. An inverted funnel (h = 7 cm, ∅ = 3 cm) in the separator collected the gas and kept the floating biomass in the reactor. Butyl rubber stoppers and tubing were used as seals.

All five 0.5 l reactors were inoculated with approximately 60 ml of granules from a 5 l UASB reactor fed 5000 mg COD/l propionate for 14 months at a liquid retention time of 10 h. Medium fed to the seed reactor was as described below, but with a twofold lower phosphate concentration. The reactors were run at 35°C in the dark.

Five reactors were operated parallel under identical conditions. At a certain time one of the reactors was stopped and granules were analysed. The remaining undisturbed reactors were run further at another propionate concentration.

The influent medium was prepared by mixing two concentrated solutions and anaerobic tap water in a mixing vessel (V= 0.3 l). In this manner the appropriate concentration of propionate was achieved. From the mixing vessel the medium was pumped into the UASB reactors at a rate of 50 ml/h. Gas bags were used to collect the biogas produced.

Medium composition

A bicarbonate buffered medium with propionate as energy and carbon source was used for the growth of granules. The final medium consisted of (g/l): KH_2PO_4 0.15, $Na_2HPO_4.2H_2O$ 0.37, $NaHCO_3$ 0.4, $Na_2S.9H_2O$ 0.048, resazurin 0.0001, sodium propionate 2.0, 4.0, 4.0, 5.9 and 1.75, respectively, (from the one stock solution) and NH_4Cl 0.3, NaCl 0.3, $CaCl_2.2H_2O$ 0.11, $MgCl_2.6H_2O$ 0.1, trace elements 1 ml/l and vitamins 1 ml/l (from the other stock solution). The trace elements solution and vitamins solution were prepared according to Zehnder et al. (1980) and Wolin et al. (1963), respectively. Because the tap water contained 0.11 g/l $Ca(HCO_3)_2$ the calcium concentration after dilution with tap water was 46.4, 40.8, 35.2, 30.0, and 30.0 mg/l for the different time intervals, respectively. Concentrated media were prepared in 10 l vessels and heat sterilized, vitamins and trace elements were filter sterilized and added aseptically. All solutions were made anaerobic by flushing with N_2/CO_2 (4/1), and the atmosphere above the media was kept anaerobic by 10 l aluminium gas bags (Tesseraux Container GmbH, Bürstadt, Germany) filled with N_2/CO_2 (4/1). The pH of the medium was 7.2.

Granule size distributions

Two independent methods were used to determine granule size distributions. The first method, described by Hulshoff Pol et al. (1986), was based on differences in sedimentation velocities of the different sized granules. Washed granules (50 - 100 ml), were allowed to settle in a 2.20 m water column. A balance, in the water column, recorded weight as a function of time. At 20°C the dynamic viscosity of water is $1.002.10^{-3}$ (Pa.s) and the density is 998.2071 (kg.m^{-3}). The density of the granules was determined from the difference in weight before and after sedimentation in the water column. If the granules are assumed to be spheres and the flow regime for the sedimentation of granules is between laminar and turbulent (1 < Re < 1000), the particle size distribution can be calculated from equations 1,2 and 3.

$$Re = 0.153 \cdot Ga^{0.71} \qquad\qquad [1]$$

$$Ga = g \cdot D_p^3 \cdot \rho_w \cdot (\rho_s - \rho_w) \cdot \mu^{-2} \qquad\qquad [2]$$

$$D_p = \frac{5.26 \cdot \mu^{0.372} \cdot \rho_w^{0.257} \cdot v^{0.885}}{(g^{0.628} \cdot (\rho_s - \rho_w)^{0.628})} \qquad\qquad [3]$$

in which Re = Reynolds number, Ga = Galileo number, g = gravity (m.s^{-2}), Dp = particle diameter (m), ρ_w = density of water (kg.m^{-3}), ρ_s = density of granules (kg.m^{-3}), μ = dynamic viscosity (Pa.s), v = sedimentation velocity (m.s^{-1}).

The second method involved image analysis. The total biomass of a reactor was well mixed, and 15 to 20 samples of 0.5 to 1 ml granular sludge were analysed. The sludge sample was mixed with 5 ml Kaiser's glycerol gelatin (Merck, Darmstadt, FRG) and poured into a petri dish ($\varnothing$ = 4.5 cm). The petri dishes were photographed with an orthochromatic film (LPD). Prints were made on 20 to 25 cm paper and overlapping particles were separated with a marker. The prints were analyzed for particle areas with a Quantimet 920 image analyzer (Cambridge Instruments, Cambridge, UK). Particle size distributions were determined using a commercial statistical software package (SAS Institute Inc., Cary, N.C.). Granules were assumed to be spheres and median diameter values were calculated. In contrast to the gravimetric method, distributions of diameter by number, were obtained. Number distributions can easily be converted to volume distributions by using the following formula:

$$V_x = n_x * 1/6 \, \pi \, d_x^3 \qquad\qquad [4]$$

in which V_x = volume of granules in interval x with diameter d_x (m^3), n_x = number of granules in interval x, d = diameter of the granules in interval x (m). In the case of a homogeneous composition of the granules, volume distributions are directly related to weight distributions.

Activity measurements
At the end of each time interval the methanogenic activity of the granular sludge with propionate and acetate was measured. Serum vials (V = 160 ml) were made anaerobic by flushing with N_2/CO_2 (4/1) and filled with 80 ml buffer solution, previously deoxygenated by boiling, and than cooled to room temperature under continuous gassing with O_2-free N_2/CO_2 (4/1). The buffer solution contained (in g per l of demineralized water): KH_2PO_4 0.41, $Na_2HPO_4.2H_2O$ 0.53, NH_4Cl 0.3, NaCl 0.3, $CaCl_2.2H_2O$ 0.1, $MgCl_2.6H_2O$ 0.1, $NaHCO_3$ 4, resazurin 0.0005. Methanogenic granular sludge (200 - 700 mg VSS) was added to the vials and then closed with butyl rubber stoppers and serum bottle caps. The vials were pressurized to 0.8 atmosphere with N_2/CO_2 (4/1) to give a pH of 7.2. To remove traces of oxygen, $Na_2S.9H_2O$ was added by syringe to obtain a final concentration of 0.24 g/l. The granular sludge samples were preincubated overnight with 0.1 to 0.2 g COD/l propionate, and then substrate from concentrated stock solutions was added to achieve final concentrations of 1.25 to 4.5 g COD/l of acetate or propionate. The vials were shaken in a water bath at 35°C for 8 to 10 h. Liquid samples were taken

with a syringe every 30 or 60 min, centrifuged and stored at -20°C for fatty acid analysis. At the end of the activity tests, the volume and dry weight of the granules were measured.

Analytical methods

Fatty acids were determined after addition of amberlite IR-120 (strong acid cation exchanger H^+-form) by gas chromatography with a Chromosorb 101 (80 - 100 mesh) column 2 m x 2 mm; temperatures (°C): column 150, injection port 220, flame ionization detector 240; carrier gas (30 ml/min): nitrogen saturated with formic acid. Fatty acids of reactor effluents were analyzed by gas chromatography with a Supelcoport (100 - 200 mesh) column coated with 10% Fluorad 431, 2 m x 4 mm; temperatures (°C): column 130, injection port 220, flame ionization detector 240; carrier gas (50 ml/min): nitrogen saturated with formic acid.

The calcium and phosphorous were determined after destruction of granular sludge in concentrated H_2SO_4 containing salicilic acid and Se (Novozamsky *et al.*, 1983). The calcium content was determined by atomic absorption spectrometry and the phosphorous content was determined colorimetrically (Schouwenburg *et al.*, 1967). Crystalline inorganic precipitates in granular sludge were qualitatively analyzed by roentgen diffraction with a Guynier camera after drying overnight at 30°C (Cullity, 1956; Anonymous, 1987). The percentage ash was determined by heating dry granules for 2 h at 600 °C.

RESULTS

Reactor performance

Granular sludge was cultivated at a selected substrate concentration over such a period of time that significant growth of biomass was obtained. The five reactors operated equally well in parallel mode, as can be seen from the increase of the sludge volume in the parallel reactors (Table 1). Only in interval 4 the high influent concentration led to a high gas production and an irregular sludge washout. Except for reactor 2, which had some lower efficiency in interval 2, the mean of the daily measured effluent concentrations of the parallel operating reactors were also in the same range (Table 1). The efficiency of the COD removal in the reactors was during the whole experiment above 95% and the effluent concentration never exceeded 200 mg COD/l.

As a consequence of growth of biomass, the sludge loading rate decreased during each time interval. An attempt was made to minimize possible effects of the sludge loading rate by chosing the time intervals such that the sludge loading rates were between 0.50 and 0.60 g COD/g VSS.d (Table 2). This strategy, however, could not be maintained for interval 5, as the influent concentration was lowered at a high biomass concentration resulting in a drop of the sludge loading rate to 0.17 g COD/g VSS.d.

During the experiment the mean biomass increase was 60.9 mg VSS/d (correlation coefficient = 0.98) and 0.95 ml sludge/d (correlation coefficient = 0.95), resulting in a mean SVI of 7.8 ml sludge/g dry wt.

Due to the dilution with tap water the calcium concentration varied between 30 and 47 mg/l, which was within the range of 6 to 150 mg Ca/l which had no effect on

granulation (Hulshoff Pol *et al.*, 1983). The calcium content increased from 0.120 g/g dry wt to 0.162 g/g dry wt whereas the phosphorous content of the granules increased from 0.073 g/g dry wt to 0.083 g/g dry wt during the course of the experiment. Roentgen analysis showed the presence of crystalline $Ca_5OH(PO_4)_3$ with an estimated diameter of 5 μm. The precipitation of $Ca_5OH(PO_4)_3$ in this system can be predicted by use of the MINEQL model (Kissel *et al.*, 1988). If all calcium is precipitated as $Ca_5OH(PO_4)_3$ this compound accounts for 60 to 90% of the ash content of the granular sludge (Table 2). In interval 5 the biomass in the effluent was found to be 0.30 gVSS in 21 days, which is only 2% of the total biomass present in the reactor.

Size distribution
Particle size distributions by number measured by the image analysis method are given in Table 3. The median diameter in interval 2 is higher than in interval 1, and is nearly equal to the median diameter of interval 3. Image analysis showed a consistent increase of median granule diameter with increasing influent concentration. A lower median diameter was observed after the influent concentration was reduced in interval 5.

The effect of the influent concentration on the median granule diameter was also found with weight analysis. Except in interval 2 the two methods gave different results. The size distribution measured by weight analysis (Table 4) showed at an increased substrate concentration in interval 2 a decrease of the median granule size. The standard deviation of the median granule diameter measured with weight analysis was 0.03 mm with a one second accuracy in time measurement. The weight analysis is less confidential compared to the image analysis, because the median granule size is dependent on the accuracy of a single time registration, whereas in image analysis the surface area of each granule is measured.

Figure 2 illustrates the size distributions of granules analyzed with both methods at interval 3 and 4. A greater number of small particles were detected by image analysis than by the gravimetric method. After conversion of diameter distribution by number obtained by image analysis to a distribution by volume, comparable median diameters were obtained.

Methanogenic activity
The methanogenic activity of the granules using acetate and propionate as test substrates was determined at the end of each time interval (Table 5). The maximal conversion rate of propionate observed in the activity measurements was always higher than the terminal loading rate in the reactors. The rate of acetate breakdown was always faster than that of propionate, acetate never accumulated in the five parallel reactors and the activity measurements. This indicates that acetate conversion is not the rate limiting step in the conversion of propionate. Activities using hydrogen as substrate were measured too. However, they were 1.4 to 30 times lower than the hydrogenotrophic activities that can be calculated from propionate conversion (results not shown). This may have resulted from transport limitation of hydrogen from the gas phase to the granules. The methanogenic activity of the sludge varied with the influent concentration. Granules grown at high influent concentrations had a high methanogenic activity on propionate and acetate, whereas the activity was low if granules were grown at low concentrations (Table 5).

TABLE 1. Mean sludge volume at the end of each time interval and mean effluent concentration with the respective standard deviations for the parallel reactors measured during the respective time intervals.

Interval	Time	Mean sludge volume	Standard deviation	Mean effluent concentration	Standard deviation
	(days)	(ml)	(%)	(mg COD/l)	(%)
0		60.1	4.7		
1	0- 49	73.7	8.8	23.6	0.23
2	50- 85	120.3	3.8	95.2	0.55
3	86-143	178.2	4.5	79.5	0.28
4	144-182	212.1	10.3	163.5	0.52
5	183-203	222.0		9.7	

TABLE 2. Characteristics of granular sludge grown in 0.5 l UASB reactors with propionate as sole carbon and energy substrate at different influent concentrations.

Interval	Time	Propionate influent	Sludge loading	Ash total	SVI	Biomass total	Calcium	Phosphorous
	(days)	(mg COD/l)	(g COD/g VSS.d)	(%)	(ml/g dry wt[1])	(g VSS[2])	(mg/g dry wt)	(mg/g dry wt)
0			n.d.[3]	49.5	12.9	2.35	120.4	72.9
1		2400						
	49		0.52	52.0	7.0	5.60	n.d.	n.d.
2		4650						
	85		0.59	55.0	5.7	9.50	132.4	73.8
3		4650						
	143		0.50	49.7	8.1	11.06	148.4	79.7
4		6900						
	182		0.59	45.4	7.4	14.03	158.8	82.8
5		2050						
	203		0.17	47.4	7.8	14.97	161.6	83.1

[1] n.d. = not determined.
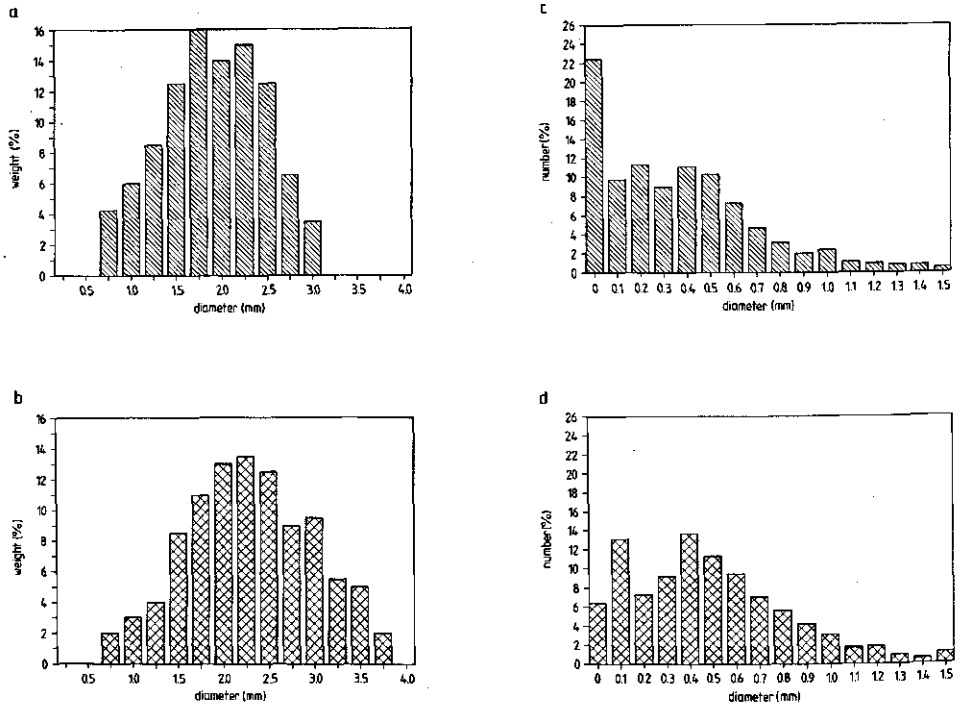[2] Dry weight (measured overnight drying at 105 °C).
[3] VSS is volatile suspended solids (dry weight - ash weight).

TABLE 3. Granule diameter distribution by number measured by image analysis at the end of five time intervals. The number of granules measured was 1984, 3583, 2064, 1419, and 1483 respectively.

Interval	1	2	3	4	5
Feed (mg COD/l)	2400	4650	4650	6900	2050
Diameter (mm)			Number (%)		
0.05-0.1	13.3	16.5	22.4	6.4	16.2
0.1-0.2	8.6	5.0	9.8	13.1	24.0
0.2-0.3	23.2	15.6	11.3	7.3	8.4
0.3-0.4	19.4	23.0	9.0	9.2	7.1
0.4-0.5	13.6	18.8	11.1	13.7	8.1
0.5-0.6	7.3	8.9	10.3	11.3	11.4
0.6-0.7	5.3	4.2	7.3	9.4	7.5
0.7-0.8	2.2	3.0	4.7	7.1	4.0
0.8-0.9	1.6	1.2	3.1	5.6	3.7
0.9-1.0	1.6	0.7	2.0	4.2	2.8
1.0-1.5	2.3	2.0	6.1	8.0	4.8
1.5-2.0	0.6	0.9	1.3	2.5	1.3
2.0-2.5	0.5	0.1	0.9	1.0	0.2
3.5-3.0	0.3	0.1	0.4	0.4	0.2
3.0-3.5	0.0	0.1	0.4	0.4	0.0
3.5-4.0	0.1	0.0	0.4	0.2	0.2
Median diameter (mm)	0.223	0.254	0.264	0.397	0.212

TABLE 4. Granule diameter distribution by weight measured by gravimetric analysis at the end of five time intervals.

Interval	1	2	3	4	5
Feed (mg COD/l)	2400	4650	4650	6900	2050
Diameter (mm)			Weight (%)		
0.25-0.50	4.0	5.5	4.25	2.0	3.5
0.50-0.75	5.5	8.75	6.0	3.0	5.5
0.75-1.00	8.0	10.75	8.5	4.0	7.0
1.00-1.25	12.0	10.75	12.5	8.5	12.0
1.25-1.50	11.0	12.25	16.0	11.0	12.0
1.50-1.75	13.5	9.5	14.0	13.0	10.5
1.75-2.00	15.5	10.5	15.0	13.5	11.5
2.00-2.25	11.0	8.0	12.5	12.5	10.0
2.25-2.50	7.0	6.5	6.5	9.0	11.0
2.50-2.75	5.0	5.0	3.5	9.5	8.0
2.75-3.00	2.5	3.0		5.5	4.5
3.00-3.25	2.5	3.0		5.0	3.0
3.25-3.50		2.0		2.0	
3.50-3.75		1.0			
Median diameter (mm)	1.64	1.53	1.79	2.15	1.97

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FIG. 2. Granule size distributions after growth of granules on 4.65 gCOD/l (interval 3) and 6.90 gCOD/l (interval 4) of propionate by weight (gravimetric analysis) (a, b) and by number (image analysis) (c, d).

TABLE 5. Actual sludge loading and potential methanogenic activities of propionate fed granular sludge using propionate and acetate as substrates at the end of each time interval. Activity measurements were made in triplicate.

End of interval	Sludge loading (µg COD propionate/g VSS.min)	Potential methanogenic activity (µg COD substrate/g VSS.min)	
		Propionate	Acetate
1	360	851 ± 22	640 ± 25
2	403	1366 ± 100	1216 ± 51
3	350	1456 ± 268	1184 ± 236
4	403	1670 ± 22	1498 ± 198
5	112	1108 ± 112	909 ± 45
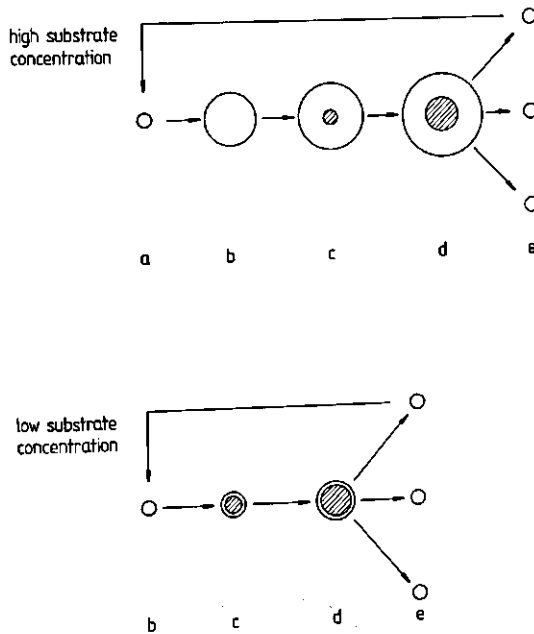
FIG. 3. Schematic representation of possible granule growth and disintegration at high and low influent concentration. (a) and (b) granule completely substrate penetrated, (c) substrate depleted area in granule core, (d) increased depleted area by granule growth, weakening of granule center due to decay, (e) granule disintegration by shear forces.

DISCUSSION

Granule size distribution was used as a characteristic parameter to determine the effect of substrate concentration on granular sludge. Results show that the particle size of methanogenic granules in UASB reactors is dependent on the influent substrate concentration. Larger granules are obtained by using high substrate concentrations, whereas low substrate concentrations lead to small granules and granule disintegration. Since the median particle size can be considered as an overall parameter for different physiological and technological conditions, measurement of the size distribution might be used for judging the quality and stability of granular sludge in full scale reactors.

Image analysis of granular sludge size distribution has several advantages over the gravimetric method. The median granule diameter in image analysis is dependent on the directly measured surface area of each individual granule, whereas a single deviation of two seconds in time registration during size distribution analysis by weight results in a 0.07 mm change of the indirectly calculated median diameter. Further, with image analysis sizes as small as 0.05 mm can be detected, whereas with the gravimetric method only particles larger than 0.5 mm are measured. Beeftink (1987) postulated from theoretical considerations that small particles should appear in high numbers and play a significant role in substrate conversion in anaerobic gas

lift reactors. The large amount of small granules in the UASB reactors may play an important role as a source of nuclei in the granulation process.

The increase in median granule size in the experiments reported here can partly be explained by growth and decay of biomass. At high influent concentrations substrate penetrates the center of the particles. The granule diameter increases until substrate depletion occurs in the core. In such substrate depleted areas, a decline in bacterial growth and finally a net decay of biomass will occur. Weakening of the granular structure in its center may be the result, leading to size reduction by shear forces. In this manner a dynamic equilibrium will be reached between growth and disintegration. A substrate shift to lower concentrations will cause larger substrate depleted areas, which finally results in a new equilibrium at a lower specific average particle size (Figure 3). A much larger relative decline in median granule diameter during interval 5 was measured by image analysis than by the gravimetric technique. This can plausibly be explained by the greater ability of the image analysis method to detect small fragments which are formed by particle disaggregation.

On the basis of a mathematical model of aggregate formation in an acidifying anaerobic gas lift reactor, Beeftink (1987) concluded that the influent concentration should not affect the size distribution of the aggregates. This may have been due to high shear forces caused by a high gas loading rate of 3500 $cm^3/cm^2.d$ in this reactor type. Although no gas was applied in the reactors described here, an apparent gas loading resulted from methane formation. The impact of gas generation on the particle size is difficult to estimate. In experiments in which the influence of extra gas supply on large granules was tested (unpublished results), a gas loading rate of 3500 $cm^3/cm^2.d$, which is roughly 5 times higher than the gas production in interval 4, resulted in a decrease of the median particle diameter of 3.34 mm to 2.73 mm (as determined by the image analyzing method).

Methanogenic activity of the granules increased steadily with increasing influent concentration and decreased after the concentration was reduced. The increase in the potential activity may be attributed to an increased fraction of viable organisms in the more heavily loaded granules. Similarly the decline in methanogenic activity after the decrease of substrate concentration may have been caused by bacterial decay. De Zeeuw (1984) found a death rate constant of 0.05 to 0.12 d^{-1} of methanogenic activity after an unfed storage of granular sludge at 30°C. Activity was only restored slowly by bacterial growth in the granular sludge.

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SUMMARY

Immobilization of anaerobic bacteria was essential for the development of high rate anaerobic systems for the treatment of waste waters. The most widely applied anaerobic reactor type in which solids retention time is uncoupled from the hydraulic retention time is the Upflow Anaerobic Sludge Blanket (UASB) reactor. In this reactor type methanogenic granular sludge is formed by self-immobilization of methanogenic consortia. The aim of the work presented in this thesis was to study microbiological aspects of the immobilization of methanogenic consortia.

To identify factors which may be of significant importance for the immobilization of anaerobic bacteria into methanogenic granular sludge a brief overview is given on the adhesion of bacteria in general as well as the microbial degradation processes that occur in anaerobic methanogenic environments (Chapter 1). Since anaerobic degradation is carried out by a series of specialized bacteria a complex biomass will result if particulate organic material or a complex medium is used as influent of an UASB reactor. To reduce the complexity of the microbial population in methanogenic granular sludge two laboratory-scale UASB reactors were fed with ethanol or propionate as substrate under defined conditions as described in Chapter 2.

The chemical and bacteriological composition of methanogenic granular sludge grown under defined conditions was studied and compared with that of methanogenic granular sludge grown on complex media (Chapter 3, 4, 5). In Chapter 3 the role of extracellular polymers in the stability of methanogenic granules was investigated. The presence of extracellular polymers with different densities and structures in methanogenic aggregates was demonstrated by electron microscopy. A three step physical disintegration procedure was used to extract water-soluble extracellular polymers from three granular sludge types. DNA was used as an intracellular marker to correct for cell lysis. Upto 3.5 mg polysaccharides/g volatile suspended solids and 5.5 mg protein/g volatile suspended solids were found to be extracellular. The amounts of extracellular polymers were much lower than reported before. However, the extracellular polymer concentration in the intermicrobial space was still high enough to form gels of sufficient strength to stabilize granules to a certain extend.

Inorganic precipitates were regularly observed by electron microscopy in methanogenic granular sludge. They may play an important role in the stability of methanogenic aggregates. Chemical analysis showed high concentrations of calcium phosphates in propionate-grown granular sludge and in granular sludge from an UASB reactor at a paper-mill. The two sludge types were used to study the effect of

calcium removal by a calcium specific chelant (EGTA) on granule stability (Chapter 4). A remarkable reduction of the granule strength of paper-mill granular sludge was found after EGTA treatment. Propionate-grown granules disintegrated completely when high EGTA concentrations were applied.

The bacteriological composition and ultrastructure of mesophilic granular sludge from a sugar refinery, ethanol-grown and propionate-grown granular sludge was studied with complementary methods (Chapter 5). The bacterial composition of the three types of granules showed that *Methanobrevibacter arboriphilus* AZ and *Methanothrix soehngenii* were the most abundant hydrogenotrophic and acetoclastic methanogens in propionate-grown sludge. *Methanospirillum hungatei* and *Methanosarcina barkeri* predominated in ethanol grown granules, whereas all types of methanogens were abundantly present in granules from a full scale reactor operated on a waste stream of a liquid sugar plant. The changes in bacterial population of the granular sludge after cultivation on ethanol or propionate could be explained by the physiological properties of the bacteria involved. With propionate as substrate a remarkable structure of two types of clusters of bacteria was observed by electron microscopical analysis. In one of these clusters, consisting of two morphological types of bacteria, one bacterial species labeled with antiserum against *Methanobrevibacter arboriphilus* AZ, whereas the other bacterial species most likely was the propionate oxidizing bacterium. The other type of cluster consisted of bundles of *Methanothrix*, which was confirmed by labeling with antiserum against *Methanothrix soehngenii*. The finding of the microbial cluster consisting of *Methanobrevibacter arboriphilus* AZ and the probable propionate oxidizing bacterium corresponded well with thermodynamic and kinetic calculations discussed in Chapter 1.

The adhesion properties of several isolates from granular methanogenic sludge and anaerobic culture collection strains were determined by measuring their hydrophobicity and electrophoretic mobility (Chapter 6). All the newly isolated bacteria were highly hydrophobic, indicating that bacteria which are immobilized in UASB reactors happen to have hydrophobic surface characteristics. The same was true for *Methanothrix soehngenii*. The abundant presence of *Methanothrix soehngenii* in methanogenic granular sludge is not only due to its high hydrophobicity but also to its high affinity for acetate and its rod shaped morphology causing that other bacteria or inorganic particles may be entangled.

An ethanol degrading homoacetogenic bacterium was isolated from ethanol adapted granular sludge. The isolated strain which differed from known homoacetogenic bacteria was named *Clostridium granularum*. The characterization and immobilization of this newly isolated homoacetogenic bacterium is described in Chapter 7. The initial immobilization of this strain from suspension into aggregates was studied in a special designed recycle UASB system. Aggregates of *Clostridium granularum* of upto 0.1 mm were formed in this system during batch operation.

Aggregate formation was enhanced when the strain was grown in the presence of a hydrogenotrophic methane bacterium.

To determine the overall effect of environmental changes in UASB reactors on the growth of granular methanogenic sludge, particle size distribution measurements were made with two independent methods namely sedimentation velocities and image analysis. With the image analysis method the presence of granules with a diameter as low as 0.05 mm could be determined, whereas the sedimentation velocity method allowed only the detection of particles larger than 0.5 mm. In Chapter 8 the mean granule diameter was used as a characteristic parameter to determine the effect of substrate concentration on granular sludge. Larger granules were obtained by using high substrate concentrations (6900 mg COD/l), whereas low substrate concentrations led to small granules through granule disintegration (2050 mg COD/l).

Conclusions

The use of single substrates for the cultivation of methanogenic granular sludge in UASB reactors allows to elucidate structure-function relationships in methanogenic granules.

The spatial orientation of bacteria in propionate grown granular sludge is a major evidence for the ecological impact of interspecies hydrogen transfer in methanogenic bacterial consortia. The presence of such spatial orientation is dependent upon the composition of the medium.

The stability of methanogenic granular sludge is dependent on the presence of extracellular polymers and inorganic precipitates. The granule size is dependent on the influent substrate concentration.

All bacteria isolated from methanogenic granular sludge were highly hydrophobic, indicating that the UASB reactor concept selects for bacteria with good adherence properties.

Since the median particle size can be considered as an overall parameter for different physiological and technological conditions, measurement of the size distribution may be used for judging the quality and stability of granular sludge in full scale reactors.

SAMENVATTING

Immobilisatie van anaërobe bakteriën was essentieel voor de ontwikkeling van een nieuwe generatie hoogbelaste waterzuiveringsreaktoren, waarin de verblijftijd van afvalwater en bakteriën ontkoppeld zijn. In het meest toegepaste nieuwe reaktorsysteem, de Upflow Anaerobic Sludge Blanket (UASB) reactor, wordt een anaërobe slibdeken in opwaartse richting doorstroomt met afvalwater. De anaërobe slibdeken in dit reaktortype bestaat uit methanogeen korrelslib dat gevormd wordt door zelf-immobilisatie van methanogene bakteriën. Het doel van deze studie was de identifikatie van belangrijke parameters die de immobilisatie van methanogene consortia beïnvloeden.

Om een inschatting te kunnen maken van de faktoren die mogelijk van groot belang zijn voor de immobilisatie van anaërobe bakteriën in methanogeen korrelslib is eerst een globaal overzicht gegeven van adhesie van bakteriën en de mikrobiologische degradatie processen die plaatsvinden in een methanogene omgeving (hoofdstuk 1). Daar bij anaërobe afbraak een grote verscheidenheid aan specialistische bakteriën betrokken is, zal er een komplexe mikrobiologische populatie in methanogeen korrelslib aanwezig zijn als er een vaste organische fraktie of een komplex vloeibaar medium als influent van een UASB gebruikt wordt. Om de komplexiteit van de mikrobiologische populatie in methanogeen korrelslib te reduceren zijn twee laboratorium UASB reaktoren gevoed met alleen ethanol of propionaat als substraat onder goed gedefinieerde omstandigheden zoals beschreven in hoofdstuk 2.

De chemische en bakteriologische samenstelling van methanogeen korrelslib, dat gegroeid was onder goed gedefinieerde omstandigheden, is bestudeerd en vergeleken met methanogeen korrelslib uit praktijk UASB-reaktoren (hoofdstuk 3, 4, 5). In hoofdstuk 3 is de rol van extracellulaire polymeren m.b.t. de stabiliteit van methanogeen korrelslib onderzocht. De aanwezigheid van extracellulaire polymeren met verschillende dichtheden en verschillende strukturen werd met elektronen-mikroskopisch onderzoek aangetoond. Tevens is getracht de kwantiteit van extracellulaire polymeren vast te stellen door korrelslib in drie stappen te desintegreren en vervolgens de wateroplosbare extracellulaire polymeren te analyseren. Tijdens deze extraktieprocedure is DNA gebruikt om korrekties uit te voeren voor eventuele celbeschadiging. Gehaltes tot 3,5 mg polysaccharide/g organische stof en 5,5 mg eiwit/g organische stof aan extracellulair materiaal werden gevonden. Deze hoeveelheden waren veel lager dan tot dusver was beschreven. Toch kunnen ook deze lage koncentraties aan extracellulaire komponenten hoog genoeg zijn om gels te vormen met voldoende sterkte om korrelslib te stabiliseren.

Anorganische neerslagen werden regelmatig met elektronen-mikroskopisch

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onderzoek waargenomen in methanogeen korrelslib. Chemische analyse toonde hoge koncentraties aan van calciumfosfaat in korrelslib dat gegroeid was met propionaat en in korrelslib uit een UASB reaktor van een papierfabriek. Beide soorten korrelslib zijn gebruikt om het effect van de calcium specifieke chelant EGTA te onderzoeken in relatie tot de stabiliteit van methanogeen korrelslib (hoofdstuk 4). Een opmerkelijke afname van de sterkte van het korrelslib uit de UASB van de papierfabriek werd waargenomen na behandeling met EGTA, terwijl een komplete desintegratie van methanogeen korrelslib optrad bij behandeling met hoge koncentraties EGTA.

De bakteriologische samenstelling en ultrastruktuur van mesofiel methanogeen korrelslib uit een UASB van een suikerfabriek, uit een met ethanol en uit een met propionaat gevoede UASB reaktor is bestudeerd met komplementaire technieken (hoofdstuk 5). Vergelijking van de bakteriologische samenstelling van de drie korreltypen liet zien dat *Methanobrevibacter arboriphilus* AZ en *Methanothrix soehngenii* de overheersende hydrogenotrofe en acetoclastische methanogene waren in propionaat gegroeid korrelslib. *Methanospirillum hungatei* en *Methanosarcina barkeri* overheersten in ethanol gegroeid korrelslib, terwijl alle typen methanogenen aanwezig waren in de korrels uit de grote schaal UASB reaktor die gevoed was met afvalwater uit een suikerfabriek. De verschuivingen in de mikrobiologische populaties van methanogeen korrelslib uit de grote schaal UASB reaktor na het kweken op ethanol en propionaat konden goed verklaard worden met de fysiologische eigenschappen van de betreffende bakteriën. Met propionaat als substraat werden bovendien opmerkelijke bakteriologische strukturen waargenomen in twee typen bakterieclusters tijdens het elektronen-mikroskopisch onderzoek. Eén van deze clusters bestond uit twee morfologische verschillende bakterie soorten. Hiervan vertoonde één bakteriesoort labeling met antiserum tegen *Methanobrevibacter arboriphilus* AZ, terwijl de andere bakteriesoort zeer waarschijnlijk een propionaat oxiderende bakterie is. Het andere bakteriecluster bestond uit bundels van *Methanothrix*, hetgeen geverifieerd is door immunolabeling met antiserum tegen *Methanothrix soehngenii*. Het bestaan van de bakteriecluster die uit *Methanobrevibacter arboriphilus* AZ en de waarschijnlijk propionaat oxiderende bacterie is samengesteld, komt goed overeen met de thermodynamische en kinetische berekeningen in hoofdstuk 1.

De adhesie eigenschappen van de verschillende bakterie isolaten uit methanogeen korrelslib en van anaërobe kollektie kulturen zijn bepaald door meting van hun hydrofobiciteit en elektroforetische mobiliteit (hoofdstuk 6). Alle nieuw geïsoleerde bakteriën waren zeer hydrofoob, hetgeen aangeeft dat geïmmobiliseerde bakteriën in UASB reaktoren hydrofobe oppervlakte eigenschappen hebben. Dit geldt ook voor *Methanothrix soehngenii*. De overheersende aanwezigheid van *Methanothrix soehngenii* in methanogeen korrelslib wordt niet alleen veroorzaakt door de hoge

hydrofobiciteit, maar ook hoge affiniteit voor acetaat en staafvormige morfologie veroorzaken dat andere bakteriën of anorganisch materiaal ingesloten worden door deze bakterie.

Een nieuwe ethanol afbrekende homoacetogene bakterie werd geïsoleerd uit methanogeen korrelslib dat geadapteerd was aan ethanol. Dit isolaat verschilde van alle tot nu toe beschreven homoacetogene bakteriën en is de naam *Clostridium granularum* gegeven. De karakterisering en immobilisatie van deze homoacetogene bakterie is beschreven in hoofdstuk 7. De initiële immobilisatie vanuit suspensie in aggregaten werd bestudeerd in een speciaal ontworpen recycle UASB systeem. Aggregaten van *Clostridium granularum* tot 0.1 mm werden in dit systeem gevormd tijdens batchgewijze cultivatie. Aggregaatvorming werd versterkt waargenomen als de kultuur gegroeid werd in aanwezigheid van hydrogenotrofe methaan bakteriën.

Om het overall effect te bepalen van milieufaktoren op de groei van methanogeen korrelslib in UASB reaktoren, zijn deeltjesgrootte verdelingen gemaakt met twee onafhankelijke methoden nl. meting van sedimentatie snelheid en beeldanalyse. Met de beeldanalyse methode konden korrels met een minimale diameter van 0,05 mm nog worden waargenomen, terwijl met meting van de sedimentatie snelheid alleen deeltjes groter dan 0,5 mm konden worden bepaald. In hoofdstuk 8 is de mediane korreldiameter gebruikt als karakteristieke parameter om het effekt te bepalen van verschillende substraat concentraties op de grootte van slib korrels. Korrels met een hogere mediane diameter werden gevonden bij een hoge substraat influent concentratie (6900 mg COD/l), terwijl bij lage substraat influent koncentraties lagere mediaan diameters werden gemeten (2050 mg COD/l).

Konklusies

Het gebruik van een enkel substraat bij kultivatie van methanogeen korrelslib in UASB reaktoren kan gebruikt worden bij de opheldering van struktuur-funktie relaties in methanogene korrels. De waargenomen ruimtelijke oriëntatie van bakteriën in propionaat gegroeid korrelslib is een belangrijke aanwijzing voor het ecologisch belang van inter species waterstof transport in methanogene bakteriële consortia. De aanwezigheid van zulke ruimtelijke strukturen is afhankelijk van de samenstelling van het medium.

De stabiliteit van methanogeen korrelslib is afhankelijk van de aanwezigheid van extracellulaire polymeren, anorganische neerslagen en de substraat influent concentratie.

Alle bakteriën die in deze studie uit methanogeen korrelslib geïsoleerd zijn, hadden allen zeer hydrofobe oppervlakte eigenschappen, hetgeen aangeeft dat het UASB reaktor concept selekteert voor bakteriën met goede hecht eigenschappen.

Daar de mediane deeltjes diameter beschouwd kan worden als een overall

parameter voor verschillende fysiologische en technologische kondities, kan de meting van de deeltjesgrootte verdeling gebruikt worden bij de beoordeling van de kwaliteit en stabiliteit van methanogeen korrelslib in praktijk reaktoren.

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Bedankt,

CURRICULUM VITAE

Johannes Theodorus Cornelis Grotenhuis is op 23 februari 1958 geboren te Den Haag. Na het behalen van het Atheneum B diploma aan de Scholengemeenschap "Simon Stevin" te Den Haag in 1976, is hij in datzelfde jaar begonnen met de studie Scheikundige Technologie aan de Technische Universiteit te Delft. In 1984 behaalde hij het diploma van scheikundig ingenieur aan deze universiteit met als hoofdvak mikrobiologie en als bijvak anorganische chemie. Het programma van de vrije studiefase omvatte o.a. de vakken biochemie, biokinetiek, biotechnologie, chemische reaktorkunde, ecologie, genetica, scheidingsprocessen, systeemtechniek, systematische anorganische chemie en anorganische materialen. Tevens werd de eerstegraads bevoegdheid tot het geven van onderwijs in de scheikunde verkregen.

In januari 1985 startte de auteur een promotie onderzoek bij de vakgroep Mikrobiologie aan de Landbouwuniversiteit te Wageningen, dat gedurende viereneenhalf jaar gefinancierd werd door de Stichting Technische Wetenschappen (STW). Momenteel is de auteur werkzaam in onderzoek naar mikrobiologische en fysisch/chemische bodemreinigingstechnieken als universitair docent bij de vakgroep Milieutechnologie aan de Landbouwuniversiteit te Wageningen. Tevens is hij werkzaam bij het Speerpuntprogramma Bodemonderzoek als sekretaris van de Programma Commissie Techniekontwikkeling Bodemonderzoek.